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(57) Abstract

The present invention relates to microbial UC pANCA antigens. The invention provides methods of diagnosing ulcerative colitis (UC) and methods of inducing tolerance in a pANCA-positive patient with UC using a histone H1-like antigen. The invention further provides methods of diagnosing UC and methods of inducing tolerance in a pANCA-positive patient with UC using a porin antigen. Methods of diagnosing UC and methods of inducing tolerance in a pANCA-positive patient with UC using a Bacteroids antigen are also provided.

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DIAGNOSIS, PREVENTION AND TREATMENT OF ULCERATIVE COLITIS, AND CLINICAL SUBTYPES THEREOF, USING MICROBIAL UC pANCA ANTIGENS

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BACKGROUND OF THE INVENTION

FIELD OF THE INVENTION

The invention relates generally to the fields of immunology and inflammatory bowel disease and more specifically to the diagnosis and treatment of a clinical subtype of ulcerative colitis.

BACKGROUND INFORMATION

Inflammatory bowel disease (IBD) is the collective term used to describe two gastrointestinal disorders of unknown etiology: Crohn's disease (CD) and ulcerative colitis (UC). The course and prognosis of ulcerative colitis, which occurs world-wide and is reported to afflict as many as two million people, varies widely. Onset of ulcerative colitis is predominantly in young adulthood with diarrhea, abdominal pain, and fever the three most common presenting symptoms. The diarrhea may range from mild to severe and often is accompanied by bleeding. Anemia and weight loss are additional common signs of UC. Ten percent to fifteen percent of all

patients with inflammatory bowel diseases such as UC will require surgery over a ten year period. In addition, patients with UC are at increased risk for the development of intestinal cancer. Reports of an increasing occurrence of psychological problems, including anxiety and depression, are perhaps not surprising symptoms of what is often a debilitating disease that strikes people in the prime of life.

Unfortunately, the available therapies for ulcerative colitis are few, and both diagnosis and treatment have been hampered by a lack of knowledge regarding the etiology of the disease. What is clear, however, is that the pathogenesis of ulcerative colitis involves immune-mediated damage to the intestinal mucosa. Autoantibodies, specifically antibodies against cytoplasmic components of neutrophils (pANCA), have been reported in 68-80% of patients with ulcerative colitis, further supporting a role for immune dysregulation in this disease. However, the antigens recognized by these pANCA autoantibodies, which would be useful in diagnosing and treating UC patients have, to date, escaped identification.

In other inflammatory bowel diseases such as Crohn's disease, bacteria have been implicated in the initiation or progression of the disease. That microbes can play a role in Crohn's disease is supported, for example, by the efficacy of antibiotics and diet in mitigating disease in some Crohn's patients. However, until now a role for microbes or microbial antigens in the immune dysregulation producing UC has not been suspected. Such microbial antigens can be the original inducers of the disease-related immune response in UC and, as such, can contain displayed B-cell epitopes that

react especially effectively with UC pANCA autoantibodies. As a consequence, these microbial antigens can increase the level of UC serodetection from its current level of 60-70% with the fixed neutrophil assay. Such microbial antigens also can bear a disease related T-cell epitope and, as likely original inducers of the disease-related immune response, can be particularly effective tolerogenic antigens for treating UC patients.

Thus, there is a need for identification and isolation of UC pANCA target antigens, including antigens of microbial origin such as those expressed in colonic bacteria of UC patients. Such antigens would be useful for diagnosing and treating the large population of UC patients that have pANCA autoantibodies. The present invention satisfies this need by providing the histone H1 UC pANCA target antigen and additional UC pANCA microbial antigens. Related advantages are provided as well.

SUMMARY OF THE INVENTION

The present invention provides methods of diagnosing ulcerative colitis (UC) by obtaining a sample from a subject suspected of having inflammatory bowel disease; contacting the sample with a histone H1-like antigen, or pANCA-reactive fragment thereof, under conditions suitable to form a complex of histone H1-like antigen, or pANCA-reactive fragment thereof, and antibody to histone H1-like antigen; and detecting the presence or absence of the complex, where the presence of the complex indicates that the subject has ulcerative colitis. A histone H1-like antigen useful in these methods can be, for example, a protein immunoreactive with NANUC-2 and

having an amino acid sequence having at least 65% amino acid identity with SEQ ID NO: 27.

The invention also provides methods of inducing tolerance in a pANCA-positive patient with UC by administering to the patient an effective dose of a histone-like H1 antigen, or tolerogenic fragment thereof. Such a histone H1-like antigen can be, for example, a protein immunoreactive with NANUC-2 and having an amino acid sequence having at least 65% amino acid identity with SEQ ID NO: 27.

The present invention also provides a composition of histone H1-like antigen, or tolerogenic fragment thereof, combined with a tolerogizing molecule. In such a composition, a histone H1-like antigen can be, for example, a protein immunoreactive with NANUC-2 and having an amino acid sequence having at least 65% amino acid identity with SEQ ID NO: 27.

In addition, the present invention provides methods of UC by obtaining a sample from a subject suspected of having inflammatory bowel disease; contacting the sample with a porin antigen, or pANCA-reactive fragment thereof, under conditions suitable to form a complex of porin antigen, or pANCA-reactive fragment thereof, and antibody to porin antigen; and detecting the presence or absence of the complex, where the presence of the complex indicates that the subject has ulcerative colitis. A porin antigen useful in these methods can be, for example, a protein immunoreactive with NANUC-2 and having at least 65% amino acid identity with SEQ ID NO: 28, SEQ ID NO: 29 or SEQ ID NO: 30. The invention also provides methods of inducing tolerance in a pANCA-positive patient with UC by

administering to the patient an effective dose of a porin antigen, or tolerogenic fragment thereof. A porin antigen useful in these methods can be, for example, a protein immunoreactive with NANUC-2 and having at least 65% amino acid identity with SEQ ID NO: 28, SEQ ID NO: 29 or SEQ ID NO: 30.

In addition, there is provided a porin antigen, or tolerogenic fragment thereof, combined with a tolerogizing molecule. In a composition of the invention, a porin antigen can be, for example, a protein immunoreactive with NANUC-2 and having at least 65% amino acid identity with SEQ ID NO: 28, SEQ ID NO: 29 or SEQ ID NO: 30.

The present invention further provides methods of diagnosing UC by obtaining a sample from a subject suspected of having inflammatory bowel disease; contacting the sample with a Bacteroides antigen, or pANCA-reactive fragment thereof, under conditions suitable to form a complex of Bacteroides antigen, or pANCA-reactive fragment thereof, and antibody to Bacteroides antigen; and detecting the presence or absence of the complex, where the presence of the complex indicates that the subject has ulcerative colitis. A Bacteroides antigen useful in these methods can be, for example, a *Bacteroides caccae* protein immunoreactive with NANUC-2 and having a molecular weight of about 100 kDa by SDS-PAGE electrophoresis.

The invention also provides methods of inducing tolerance in a pANCA-positive patient with UC by administering to the patient an effective dose of a Bacteroides antigen, or tolerogenic fragment thereof. Such a Bacteroides antigen useful in these methods can

be, for example, a *Bacteroides caccae* protein immunoreactive with NANUC-2 and having a molecular weight of about 100 kDa by SDS-PAGE electrophoresis.

Additionally, the invention provides a composition containing a Bacteroides antigen, or a tolerogenic fragment thereof, combined with a tolerogizing molecule. A *Bacteroides caccae* protein immunoreactive with NANUC-2 and having a molecular weight of about 100 kDa by SDS-PAGE electrophoresis is an example of a Bacteroides antigen useful in a composition of the invention.

Further provided herein are methods for identifying an agent useful for treating UC. The methods involve obtaining a sample of enteric bacteria from a patient with UC; isolating from the sample a bacterial species that expresses a pANCA-reactive antigen; contacting the bacterial species with an agent; and assaying for the reduced growth or viability of the bacterial species, where the reduced growth or viability of the bacterial species indicates that the agent is an agent useful for treating UC. Enteric bacterial species useful in these methods can be, for example, from the genera of Mycobacteria, Escherichia or Bacteroides.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the amino acid sequences of human histone H1 isoforms $H1^{s}$-1, $H1^{s}$-2, $H1^{s}$-3, $H1^{s}$-4, H1° and H1t.

Figure 2 shows Western analysis with a representative pANCA monoclonal, NANUC-2. Protein samples represent a HL60 cell nuclear fraction, purified

calf thymus histones and histones purified from human neutrophils (PMN). The perchloric acid (PCA) insoluble PMN fraction contains the core histones, while the perchloric acid soluble fraction contains histone H1.

Figure 3 shows enzyme-linked immunosorbent assay analysis of NANUC-1 and NANUC-2 with neutrophil (PMN), total calf thymus histones (histone), purified calf thymus histone H1 (H1) or tetanus toxoid antigen (TT).

Figure 4 shows Western analysis of whole cell, nuclear and cytoplasmic fractions of Molt-4 cells and human neutrophils. Identical blots were reacted with NANUC-1 or NANUC-2 or with negative control anti-tetanus toxoid antibody.

Figure 5 shows the reactivity of histone H1 derived peptides with the NANUC-1 and NANUC-2 antibodies.

Figure 6 shows the growth conditions and media used to culture seven Mycobacterial strains: *M. tuberculosis; M. bovis; M. bovis* BCG; *M. smegmatis 1-2c; M. avium; M. avium paratuberculosis* and *M. avium paratuberculosis* "Linda" strain.

Figure 7 shows Western analysis of seven Mycobacterial strains with the NANUC-2 antibody. Each of the seven strains express a pANCA-reactive protein of 30-32 kDa.

Figure 8 shows the alignment of an N-terminal fragment of a *M. avium paratuberculosis* histone H1-like antigen (SEQ ID NO: 31; designated "N-term"); a predicted protein of 214 amino acids from the *M. tuberculosis*

genome (SEQ ID NO: 27; designated "214"); and human histone H1 isoform H1.5 (SEQ ID NO: 32; designated "H1.5").

Figure 9 shows Western analysis of UC patient colonic bacterial isolates with the NANUC-2 antibody. A. Whole cell extracts of four *E. coli* isolates (designated P1Bc5, P1Bc9, P2c2 and P2c5). B. Whole cell extracts from *E. coli* isolate P2Lc2 and two *Bacteroides caccae* isolates (designated P2Lc3 and P2Lc5).

Figure 10 shows Western analysis of several different *Bacteroides* CD patient colonic isolates. A and B. Whole cell extracts were immunoreacted with NANUC-2 antibody.

Figure 11 shows the amino acid sequences of human histone H1.5 (SEQ ID NO: 32); a 214 amino acid *M. tuberculosis* histone H1-like antigen (SEQ ID NO: 27); a 323 amino acid outer membrane protein F precursor from *E. coli* (SEQ ID NO: 28); a 377 amino acid outer membrane protein F precursor from *E. coli* (SEQ ID NO: 29); and a 367 amino acid outer membrane protein c precursor from *E. coli* (SEQ ID NO: 30).

Figure 12 shows the alignment of 19 amino acid sequence which is common to two *E. coli* porin antigens (SEQ ID NO: 33; designated "N-term"); the 323 amino acid outer membrane protein F precursor from *E. coli* (SEQ ID NO: 28; designated "323"); and human histone H1 isoform H1.5 (SEQ ID NO: 32; designated "H1.5").

DETAILED DESCRIPTION OF THE INVENTION

Perinuclear anti-neutrophil cytoplasmic antibodies (pANCA) are present in the sera of most patients with ulcerative colitis (UC), and are a familial trait associated with disease susceptibility and disease-associated MHC haplotypes. Although sera from pANCA-positive ulcerative colitis patients are known to react with a component of neutrophils, the antigen responsible for the UC pANCA reactivity has long eluded identification. The present invention is directed to the exciting discovery that the pANCA autoantibody present in the sera of most patients with UC reacts with histone H1. The present invention further is directed to the discovery that UC pANCA also reacts with antigens of microbial origin. In particular, disclosed herein are a pANCA-reactive histone H1-like antigen, porin antigen and 100 kDa antigen selectively expressed in Bacteroides, each of which is a microbial antigen useful in the diagnosis, prevention and treatment of ulcerative colitis.

As disclosed herein, Western analysis demonstrates that a nuclear protein doublet of 32-33 kDa from neutrophils is specifically reactive with a representative UC pANCA monoclonal antibody, NANUC-2 (see Figure 2). Purification and protein sequencing of the NANUC-2 reactive protein doublet identified the UC pANCA target antigen as histone H1. Specific binding of NANUC-2 to histone H1 was confirmed using purified human neutrophil histone H1 and purified calf thymus histone H1. Identification of histone H1 as a UC pANCA target antigen provides a valuable reagent for diagnosing the presence of pANCA in UC patients and for ameliorating the abnormal immune process involved in ulcerative colitis.

Thus, the invention is directed to methods for diagnosing a pANCA-positive clinical subtype of UC and determining susceptibility to UC using histone H1. The invention also is directed to methods of treating UC by inducing tolerance in a pANCA-positive UC patient and preventing UC in a healthy individual by administering the recently identified UC pANCA target antigen, histone H1.

The present invention provides methods of diagnosing UC by obtaining a sample from a subject suspected of having inflammatory bowel disease; contacting the sample with human histone H1, or pANCA-reactive fragment thereof, under conditions suitable to form a complex of human histone H1, or pANCA-reactive fragment thereof, and antibody to human histone H1; and detecting the presence or absence of the complex, where the presence of the complex indicates that the subject has ulcerative colitis. Human histone H1 useful in these methods can be, for example, histone H1 isoform $H1^s$-2 or a pANCA-reactive fragment thereof such as SEQ ID NO: 7, SEQ ID NO: 13, SEQ ID NO: 14 or SEQ ID NO: 20.

The present invention also provides methods of diagnosing a pANCA-positive clinical subtype of ulcerative colitis in a patient with UC. A pANCA-positive clinical subtype of UC can be diagnosed, for example, by obtaining a sample from a patient with UC; contacting the sample with human histone H1, or pANCA-reactive fragment thereof, under conditions suitable to form a complex of human histone H1, or pANCA-reactive fragment thereof, and antibody to human histone H1; and detecting the presence or absence of the complex, where the presence of the complex indicates that the patient has the pANCA-positive clinical subtype of

UC. A pANCA-positive clinical subtype of UC also can be diagnosed by obtaining a sample from a patient with UC; contacting the sample with purified histone H1 isoform $H1^s$-2, or pANCA-reactive fragment thereof, under conditions suitable to form a complex of histone H1 isoform $H1^s$-2, or pANCA-reactive fragment thereof, and antibody to histone H1 isoform $H1^s$-2; and detecting the presence or absence of the complex, where the presence of the complex indicates that the patient has the pANCA-positive clinical subtype of UC.

The methods of the invention relate to diagnosing and treating UC, which is a disease of the large intestine characterized by chronic diarrhea with cramping abdominal pain, rectal bleeding, and loose discharges of blood, pus and mucus. The manifestations of this disease vary widely. A pattern of exacerbations and remissions typifies the clinical course of most UC patients (70%), although continuous symptoms without remission are present in some patients with UC. Local and systemic complications of UC include arthritis, eye inflammation such as uveitis, skin ulcers and liver disease. In addition, ulcerative colitis and especially long-standing, extensive disease is associated with an increased risk of colon carcinoma.

Several pathologic features characterize UC in distinction to other inflammatory bowel diseases. Ulcerative colitis is a diffuse disease that usually extends from the most distal part of the rectum for a variable distance proximally. The term left-sided colitis describes an inflammation that involves the distal portion of the colon, extending as far as the splenic flexure. Sparing of the rectum or involvement of the right side (proximal portion) of the colon alone is

unusual in ulcerative colitis. Furthermore, the inflammatory process of UC is limited to the colon and does not involve, for example, the small intestine, stomach or esophagus. In addition, ulcerative colitis is distinguished by a superficial inflammation of the mucosa that generally spares the deeper layers of the bowel wall. Crypt abscesses, in which degenerate intestinal crypts are filled with neutrophils, also are typical of the pathology of ulcerative colitis (Rubin and Farber, Pathology (Second Edition) Philadelphia: J.B. Lippincott Company (1994), which is incorporated herein by reference).

As used herein, the term "ulcerative colitis" is synonymous with "UC" and means a disease having clinical features of left-sided colonic disease accompanied by a characteristic endoscopic or histopathologic feature of UC. Clinical features of left-sided colonic disease, as used herein, are rectal bleeding, urgency and tenesmus. The rectal bleeding may be accompanied by mucus discharge. Additional clinical features that may be present in UC include treatment with topical therapy and recommended or performed total or near-total colectomy.

A characteristic endoscopic feature of UC, which when present with clinical features of left-sided colonic disease indicates ulcerative colitis, is inflammation that is more severe distally than proximally or continuous inflammation. Additional typical endoscopic features that may be present in UC include inflammation extending proximally from the rectum or shallow ulcerations or the lack of deep ulcerations.

A characteristic histopathologic feature of UC, which when present with clinical features of left-sided colonic disease indicates ulcerative colitis, is homogeneous, continuous, predominantly superficial inflammation or a lack of "focality" within biopsy specimens. Additional typical histopathologic features that may be present in UC include the presence of crypt abscesses or a lack of granulomas. Characteristic clinical features of left-sided colonic disease and characteristic endoscopic and histopathologic features of ulcerative colitis are summarized in Table 1.

As used herein, the term "subject suspected of having inflammatory bowel disease" means any animal capable of having ulcerative colitis, including a human, non-human primate, rabbit, rat or mouse, especially a human, and having one or more symptoms of ulcerative colitis or Crohn's disease as described hereinabove.

As used herein, the term "patient with UC" means a patient having ulcerative colitis, as defined by the presence of clinical features of left-sided colonic disease accompanied by a characteristic endoscopic or histopathologic feature of UC as defined herein.

The pathogenesis of ulcerative colitis, although poorly understood, ultimately involves immune-mediated tissue damage. Ulcerative colitis is associated with various immunologic abnormalities, many of which can be secondary to inflammation. Similar to autoimmune disorders such as diabetes mellitus and multiple sclerosis, ulcerative colitis can represent a process of immune dysfunction directed against intrinsic intestinal mucosa cells. However, ulcerative colitis occurs in a mucosal site interfacing with the intestinal

lumen. Thus, a primary immune target also can be an extrinsic agent such as a chronic microbial colonist. In this case, the mucosal injury characteristic of UC is a consequence of inflammatory bystander damage to resident parenchymal cells.

Table 1

A. Clinical features of left-sided colonic disease	1. Rectal bleeding possibly accompanied by mucus discharge 2. Urgency 3. Tenesmus 4. Treatment with topical therapy 5. Recommended or performed total or near-total colectomy
B. Endoscopic features of UC	6. Inflammation that is more severe distally than proximally 7. Continuous inflammation 8. Inflammation extending proximally from the rectum 9. Shallow ulcerations or lack of deep ulcerations
C. Histopathologic features of UC	10. Homogeneous, continuous, predominantly superficial inflammation 11. Lack of "focality" within biopsy specimens 12. Crypt abscesses 13. Lack of granulomas

Host genetic factors can confer susceptibility or resistance to tissue damage elicited by a chronic local immune response. For example, IBD is associated with polymorphisms in MHC class II, ICAM-1 and TNF-α loci (Yang et al., J. Clin. Invest. 92:1080-1084 (1993)), and animal and clinical studies directly implicate TNF levels in disease. In the case of autoimmune diseases where the primary target is a self-antigen, host genetic factors can play a role in disease by controlling, for example, T-cell clonal abundance, peptide antigen presentation, and levels of cytokines modulating different effector responses. Host genetic diversity also can affect variable susceptibility to microbial organisms. Thus, pathogenesis of ulcerative colitis can result from a primary abnormality of the immune system, or from an initial injury by an infectious agent that is perpetuated through immune-mediated or other processes.

Certain immune-mediated disorders, including systemic lupus erythematosis, primary biliary cirrhosis and autoimmune hepatitis, are closely associated with distinctive patterns of autoantibody production. In the case of ulcerative colitis, anti-neutrophil cytoplasmic antibodies that produce a perinuclear staining pattern (pANCA) are elevated in 68-80% of UC patients and less frequently in other disorders of the colon. Serum titers of ANCA are elevated regardless of clinical status and, thus, do not reflect disease activity. High levels of serum ANCA also persist in patients five years post-colectomy. Although pANCA is found only very rarely in healthy adults and children, healthy relatives of UC patients have an increased frequency of pANCA, indicating that pANCA may be an immunogenetic susceptibility marker.

Serum antibodies to cytoplasmic components of a neutrophil (ANCA) can be detected, for example, using indirect immunofluorescence microscopy of alcohol-fixed neutrophils. ANCA activity has been divided into two broad categories: cytoplasmic neutrophil staining (cANCA) and perinuclear to nuclear staining or cytoplasmic staining with perinuclear highlighting (pANCA). As used herein, the term "perinuclear anti-neutrophil cytoplasmic antibody" is synonymous with "pANCA" and means an antibody that reacts specifically with a neutrophil to give perinuclear to nuclear staining or cytoplasmic staining with perinuclear highlighting.

The term "clinical subtype of UC," as used herein, means a subgroup of patients having ulcerative colitis whose features of disease are more similar to each other than to other patients with ulcerative colitis. The term "pANCA-positive clinical subtype of UC" means that subgroup of UC patients having pANCA.

Serum anti-neutrophil cytoplasmic antibodies previously have been used to characterize clinically distinct subsets of UC patients. For example, the presence of pANCA has been associated with treatment-resistant left-sided ulcerative colitis; aggressive UC (Vecchi et al., Digestion 55:34-39 (1994)); the requirement for surgery early in the course of UC (Boerr et al., Gastroenterol. 108: A785 (1995)) or development of pouchitis following ileal pouch-anal anastomosis for UC (Sandborn et al., Gastroenterol. 104: A774 (1993); Patel et al., Br. J. Surg. 81:724-726 (1994); Vecchi et al., Lancet 344:886-887 (1994); Sandborn et al., Am. J. Gastroenterol. 90:740-747 (1995)). Thus, the ability to identify a pANCA-positive clinical subtype of UC can be useful in predicting, for

example, treatment-resistant UC; the progression of UC; the need for early surgery or the development of pouchitis.

The present invention is directed to the surprising discovery that an antigen that reacts with pANCA of ulcerative colitis is histone H1. Thus, the UC pANCA target antigen is a member of the histone family, which are highly-conserved proteins characterized by basic residues that contact the negatively charged phosphate groups in DNA and organize the DNA of eukaryotes into chromatin. Histones H2A, H2B, H3 and H4 are the core histones that make up nucleosomes, while histone H1, which is associated with nucleosomes at a 1 to 1 ratio, is required for higher order chromatin structure.

Histone H1 has a conserved central globular domain with extended, flexible N- and C-terminal domains. Sites of reversible chemical modification, such as phosphorylation and acetylation, occur in these extended N- and C-terminal regions and can regulate histone-DNA interactions (Bradbury et al., Bioessays 14: 9-16 (1992)). The histone H1 family has multiple isoforms including $H1^{s}$-1, $H1^{s}$-2, $H1^{s}$-3 and $H1^{s}$-4, which are present in all normal somatic cells; the highly variable $H1^{o}$ isoform, which is associated with differentiated cell types; and the testis-specific isoform H1t (Parseghian et al., Protein Sci. 3:575-587 (1994), which is incorporated herein by reference). That functional differences can be associated with alternative histone H1 isoforms is supported by differences among the isoforms in the time of protein synthesis; turnover rate; amount and pattern of phosphorylation and ability to condense DNA *in vitro*.

As used herein, the term "histone H1" means one or more proteins having at least about 80% amino acid identity with at least one amino acid sequence of human histone H1 isoform $H1^s$-1 (SEQ ID NO: 1); $H1^s$-2 (SEQ ID NO: 2); $H1^s$-3 (SEQ ID NO: 3); $H1^s$-4 (SEQ ID NO: 4); $H1^o$ (SEQ ID NO: 5); H1t (SEQ ID NO: 6). Thus, the term histone H1 encompasses, for example, one or more of the human histone H1 isoforms having an amino acid sequence shown in Figure 1.

The term histone H1 also encompasses one or more non-human histone H1 isoforms having at least about 80% amino acid identity with at least one of the human histone H1 isoforms having an amino acid sequence shown in Figure 1. For example, the term histone H1 encompasses, for example, a mouse or bovine $H1^s$-1 isoform having an amino acid sequence described in Parseghian et al., *supra*, 1994, or a rabbit or bovine $H1^s$-2 isoform having an amino acid sequence described in Parseghian et al., *supra*, 1994. Similarly, the term histone H1 encompasses, for example, a rabbit or rat $H1^s$-3 isoform having an amino acid sequence described in Parseghian et al., *supra*, 1994, or a mouse, rat, rabbit or bovine $H1^s$-4 isoform having an amino acid sequence described in Parseghian et al., *supra*, 1994. As disclosed herein, histone H1 useful in the invention can be obtained from a variety of species. For example, histone H1 that forms a complex with a representative UC monoclonal antibody (NANUC-2) and, thus, can form a complex with pANCA, can be purified from human neutrophil or calf thymus as described in Example I.

As used herein, the term "human histone H1" means one or more proteins having at least one amino acid sequence of human histone H1 isoform $H1^s$-1 (SEQ ID NO: 1);

$H1^s$-2 (SEQ ID NO: 2); $H1^s$-3 (SEQ ID NO: 3); $H1^s$-4 (SEQ ID NO: 4); H1° (SEQ ID NO: 5) or H1t (SEQ ID NO: 6).

As used herein, the term "histone H1 isoform $H1^s$-3" is synonymous with "$H1^s$-3" and means a protein having at least about 80% amino acid identity with the amino acid sequence of human histone isoform $H1^s$-3 (SEQ ID NO: 3) shown in Figure 1. For example, the term $H1^s$-3 encompasses human histone H1 isoform $H1^s$-3 having the amino acid sequence (SEQ ID NO: 3) shown in Figure 1. The term $H1^s$-3 also encompasses a non-human $H1^s$-3 protein having at least about 80% amino acid identity with the amino acid sequence shown as SEQ ID NO: 3, such as a rabbit or rat $H1^s$-3 isoform having an amino acid sequence described in Parseghian et al., *supra*, 1994.

As used herein, the term "histone H1 isoform $H1^s$-2" is synonymous with "$H1^s$-2" and means a protein having at least about 80% amino acid identity with the amino acid sequence of human histone isoform $H1^s$-2 (SEQ ID NO: 2) shown in Figure 1. For example, the term $H1^s$-2 encompasses human histone H1 isoform $H1^s$-2 having the amino acid sequence (SEQ ID NO: 2) shown in Figure 1. The term $H1^s$-2 also encompasses a non-human $H1^s$-2 protein having at least about 80% amino acid identity with the amino acid sequence shown as SEQ ID NO: 2, such as a rat or bovine $H1^s$-2 isoform having an amino acid sequence described in Parseghian et al., *supra*, 1994.

As further disclosed herein, Western analysis demonstrates that an antigen of 30-32 kDa present in various species of Mycobacteria is specifically reactive with NANUC-2, a representative UC pANCA monoclonal antibody (see Figure 7). Isolation and amino-terminal sequencing of the 30-32 kDa protein from *M. avium*

paratuberculosis revealed that the amino-terminal sequence was nearly identical to the amino-terminal sequence of a predicted *M. tuberculosis* protein of 214 amino acids (SEQ ID NO: 27) designated HupB. This *M. tuberculosis* protein shares 48% sequence similarity with human histone H1 isoform H1.5 (SEQ ID NO: 32) (see Figure 8), indicating that the 214 amino acid *M. tuberculosis* protein is a histone H1-like antigen. As disclosed in Example VB, immunoreactivity of recombinant HupB (SEQ ID NO: 27) with NANUC-2 confirmed that HupB is a UC pANCA antigen and localized the NANUC-2 binding epitope to the 90 C-terminal amino acids of this antigen (residues 125 to 214 of SEQ ID NO: 27).

Identification of a pANCA-reactive Mycobacterial histone H1-like antigen implicates Mycobacteria in the pathogenesis of UC and provides a valuable reagent for diagnosing or ameliorating UC. Based on this finding, the present invention provides methods for diagnosing UC or a pANCA-positive clinical subtype of UC as well as methods of determining susceptibility to UC. The present invention also provides methods of treating UC and methods of preventing UC in a healthy individual by administering the recently identified UC pANCA target antigen, a histone H1-like antigen.

In particular, the present invention provides methods of diagnosing UC by obtaining a sample from a subject suspected of having inflammatory bowel disease; contacting the sample with a histone H1-like antigen, or pANCA-reactive fragment thereof, under conditions suitable to form a complex of the histone H1-like antigen, or pANCA-reactive fragment thereof, and antibody to the histone H1-like antigen; and detecting the

presence or absence of the complex, where the presence of the complex indicates that the subject has ulcerative colitis. A histone H1-like antigen useful in these methods can be, for example, a protein immunoreactive with NANUC-2 and having at least 65% amino acid identity with SEQ ID NO: 27.

The present invention also provides methods of diagnosing a pANCA-positive clinical subtype of ulcerative colitis in a patient with UC. A pANCA-positive clinical subtype of UC can be diagnosed, for example, by obtaining a sample from a patient with UC; contacting the sample with a histone H1-like antigen, or pANCA-reactive fragment thereof, under conditions suitable to form a complex of the histone H1-like antigen, or pANCA-reactive fragment thereof, and antibody to histone H1-like antigen; and detecting the presence or absence of the complex, where the presence of the complex indicates that the patient has the pANCA-positive clinical subtype of UC.

As used herein, the term "histone H1-like antigen" means a pANCA-reactive protein having linear or conformational homology to histone H1. A histone H1-like antigen generally is characterized as having at least about 48% amino acid similarity with one or more of the following human histone H1 isoforms: $H1^{s}$-1 (SEQ ID NO: 1); $H1^{s}$-2 (SEQ ID NO: 2); $H1^{s}$-3 (SEQ ID NO: 3); $H1^{s}$-4 (SEQ ID NO: 4); $H1^{\circ}$ (SEQ ID NO: 5); or H1t (SEQ ID NO: 6).

The term "histone H1-like antigen" encompasses, for example, a microbial histone H1-like antigen, which is a histone H1-like antigen of microbial origin having a molecular weight of about 30-32 kDa on SDS-PAGE analysis. An example of a microbial histone H1-like antigen is a

30-32 kDa protein which is immunoreactive with NANUC-2 and is isolated from a Mycobacteria such as *M. tuberculosis; M. bovis,* and *M. bovis* BCG; *M. smegmatis 1-2c; M. avium; M. avium paratuberculosis* and *M. avium paratuberculosis* "Linda" strain (see Figure 7). A microbial histone H1-like antigen can be, for example, the *M. tuberculosis* histone H1-like protein (SEQ ID NO: 27).

A histone H1-like antigen particularly useful in the invention is the *M. tuberculosis* histone H1-like protein (SEQ ID NO: 27), designated HupB by the research consortium for the *M. tuberculosis* genome (Cole et al., Nature 393:537-544 (1998)). The sequence of the 214 amino acid *M. tuberculosis* antigen (HupB; SEQ ID NO: 27) was compared with other DNA binding proteins in available databases: the antigen shares amino acid homology with bacterial HU binding proteins as well as histone H1. The HU similarity was localized to the amino-terminal half of the *M. tuberculosis* histone H1-like protein (HupB; SEQ ID NO: 27), while histone H1 similarity was localized to the carboxy-terminal half, indicating that the *M. tuberculosis* protein originated from a fusion between a prokaryotic HU type protein and histone H1.

The sequence of the 214 amino acid *M. tuberculosis* antigen (HupB; SEQ ID NO: 27) varies significantly from mammalian histone H1, being more closely related to the histone H1-like proteins of more primitive life forms. Furthermore, bacterial DNA binding motifs (PAKKAA; SEQ ID NO: 42) are expressed extensively at the carboxy terminus, while histone H1 carboxy terminal specific motifs (SPKKAK: SEQ ID NO: 43) were not present (Allan et al., Nature 288:675-679 (1980); Hayes

et al., J. Biochem. 271:25817-25822 (1996); Pruss et al., Science 274:614-617 (1996)). Histone H1 SPKKAK (SEQ ID NO: 43) motifs have been implicated in linker DNA binding and in post-translational regulation of histone H1 activity in formation and stabilization of packed chromatin. Such motifs are highly conserved in higher organisms (Churchill and Suzuki, EMBO J. 8:4189-4195 (1989); Suzuki et al., J. Biochem. 108:356-364 (1990); Suzuki et al.; J. Biochem. 208:347-355 (1990)). Thus, HupB (SEQ ID NO: 27) may have originated early in evolution rather than by gene capture from a mammalian host.

The 214 amino acid *M. tuberculosis* antigen (HupB; SEQ ID NO: 27) can play a role in UC pathogenesis by altering host transcription profiles. The function of histone H1 as a transcriptional regulator is well established (Shen and Gorovsky, Cell 86:475-483 (1996); Brown et al., Nuc. Acids Res. 25:5003-5009 (1997); Juan et al., EMBO J. 13:6031-6040 (1994); Juan et al., J. Biol. Chem. 272:3635-3640 (1997); and Schultz et al., J. Biol. Chem. 271:25742-25745 (1996)). Furthermore, changes in *E. coli* growth characteristics mediated by HupB (SEQ ID NO: 27) can be induced at the level of transcription (Scarlato et al., Mol. Microbiol. 15:871-881 (1995); and Zu et al., J. Bacteriol. 178:2982-2985 (1996)).

The term histone H1-like antigen encompasses a protein that has at least 65% amino acid identity with the 214 amino acid *M. tuberculosis* histone H1-like antigen (HupB; SEQ ID NO: 27). In particular, a histone H1-like antigen can have an amino acid sequence having, for example, at least 70%, 80%, 85%, 90% or 95% amino acid identity with SEQ ID NO: 27.

A histone H1-like antigen can be isolated from a variety of eukaryotic and prokaryotic organisms, including bacteria such as Mycobacteria and others. A histone H1-like antigen can be isolated from a variety of species of Mycobacteria, including *Mycobacterium tuberculosis, Mycobacterium bovis, Mycobacterium smegmatis 1-2c, Mycobacterium avium* and *Mycobacterium avium paratuberculosis*. Histone H1-like antigens isolated from a variety of Mycobacterial species are disclosed in Example V.

The present invention also relates to a UC pANCA porin antigen expressed by enteric bacteria of UC patients. As disclosed in Example VI, Western analysis demonstrates that a 35 kDa protein isolated from colonic *E. coli* of UC patients is specifically reactive with the UC pANCA monoclonal antibody NANUC-2 (see Figure 9). The larger and smaller of two pANCA-reactive *E. coli* proteins were isolated and each sequenced from the N-terminus, revealing identical N-terminal sequence (SEQ ID NO: 33). This amino-terminal sequence indicated that the two pANCA-reactive *E. coli* proteins were related to the outer membrane proteins F and C (OmpF and OmpC; see Figure 11). As disclosed in Example VIB, the pANCA-reactive 35 kDa *E. coli* protein is absent from mutant strains that do not express OmpC, indicating that a UC pANCA porin antigen can be OmpC. As shown in Figure 12, the porin antigen of the invention lacks detectable linear sequence homology with histone H1 but can have conformational sequence homology with one or more pANCA-reactive epitopes of histone H1. These results indicate that microbial antigens lacking detectable linear sequence homology to histone H1 can be expressed by enteric colonic bacteria in UC patients and can play a role in the immune dysregulation in UC.

Isolation of the porin antigen disclosed herein provides a novel UC pANCA target antigen for diagnosing and treating ulcerative colitis. Based on this discovery, the invention provides methods for diagnosing UC or a pANCA-positive clinical subtype of UC and methods of determining susceptibility to UC using a porin antigen. The invention further provides methods of treating UC by inducing tolerance in a pANCA-positive UC patient and methods of preventing UC in a healthy individual by administering a porin antigen.

In particular, the present invention provides a method of diagnosing UC by obtaining a sample from a subject suspected of having inflammatory bowel disease; contacting the sample with a porin antigen, or pANCA-reactive fragment thereof, under conditions suitable to form a complex of the porin antigen, or pANCA-reactive fragment thereof, and antibody to the porin antigen; and detecting the presence or absence of the complex, where the presence of the complex indicates that the subject has ulcerative colitis. A porin antigen useful in these methods can be, for example, a protein immunoreactive with NANUC-2 and having an amino acid sequence having at least 65% amino acid identity with SEQ ID NO: 28, SEQ ID NO: 29 or SEQ ID NO: 30.

The present invention also provides methods of diagnosing a pANCA-positive clinical subtype of ulcerative colitis in a patient with UC. A pANCA-positive clinical subtype of UC can be diagnosed, for example, by obtaining a sample from a patient with UC; contacting the sample with a porin antigen, or pANCA-reactive fragment thereof, under conditions suitable to form a complex of the porin antigen, or pANCA-reactive fragment thereof, and antibody to porin

antigen; and detecting the presence or absence of the complex, where the presence of the complex indicates that the patient has the pANCA-positive clinical subtype of UC.

The porin antigens disclosed herein belong to a class of transmembrane proteins that are found in the outer membranes of bacteria, including gram-negative, enteric bacteria such as *E. coli*. The porins in the outer membrane of an *E. coli* cell provide channels for passage of disaccharides, phosphate and similar molecules. Porins can be trimers of identical subunits arranged to form a barrel-shaped structure with a pore at the center (Lodish et al., Molecular Cell Biology, Chapter 14 (1995), which is incorporated herein by reference).

Two major porin proteins found in the outer membranes of bacteria such as *E. coli* are outer-membrane protein F ("OmpF") and outer-membrane protein C ("OmpC"). These two porins are similar in structure and function, assembling as trimers in the outer membrane to form aqueous channels that allow the passive diffusion of small, hydrophilic molecules across the hydrophobic barrier. The diameters of the OmpF and OmpC pores differ with the pore of OmpF being 1.2 nm while the diameter of the OmpC pore is 1.1 nm. This difference results in a faster rate of diffusion through the OmpF pores than through the OmpC pores.

Porin expression can be influenced by environmental conditions, including osmolarity, temperature, growth phase and toxin concentration. For example, in the intestine, where both nutrient and toxic molecule concentrations are relatively high, OmpC, with a

smaller pore diameter, is the predominant porin (Pratt et al., Mol. Micro., 20:911-917 (1996), which is incorporated herein by reference).

As used herein, the term "porin antigen" means a pANCA-reactive protein that has linear or conformational homology to OmpF, OmpC or another *E. coli* porin. A porin antigen generally is a protein that, in nature, forms a trimeric structure in the outer membrane of bacteria that allows the passage of small molecules, or a precursor of such a protein. A porin antigen can be derived from a gram-negative bacterium, such as *E. coli*, and can be, for example, OmpF or OmpC, or a homolog thereof.

The term "porin antigen," as used herein, encompasses a protein that has at least 65% amino acid identity with one or more of the following proteins: SEQ ID NO: 28, SEQ ID NO: 29 or SEQ ID NO: 30. A porin antigen can have, for example, at least 70%, 80%, 85%, 90% or 95% amino acid identity with SEQ ID NO: 28, SEQ ID NO: 29 or SEQ ID NO: 30.

An "ompC porin antigen" can be particularly useful in the invention. An "ompC porin antigen," as defined herein, is a protein that has at least 65% amino acid identity with the ompC amino acid sequence shown as SEQ ID NO: 30. An ompC porin antigen can have, for example, at least 70%, 80%, 85%, 90% or 95% amino acid identity with the OmpC amino acid sequence shown as SEQ ID NO: 30.

The present invention also relates to a novel UC pANCA target antigen derived from Bacteroides. Bacteroides is a genus of gram-negative, anaerobic,

nonspore-forming, rod-shaped bacteria. They are normal inhabitants of the intestinal tract, and may constitute the predominant bacteria of the normal human colon. Some species of Bacteroides are known to be pathogenic, causing serious abscesses and bacteriomas.

As disclosed herein, an antigen of about 100 kDa isolated from extracts of *Bacteroides caccae* is specifically reactive with NANUC-2 (see Figure 9B). This pANCA-reactive antigen is selectively expressed in Bacteroides and is not expressed in all bacteria, for example, in *E. coli* (see Figure 9; compare lanes 2 and 3 of panel B with other lanes). Identification of a Bacteroides antigen as a UC pANCA target antigen provides an additional valuable reagent for diagnosing the presence of pANCA in UC patients and for treating the disease. The Bacteroides antigen disclosed herein can be used alone for diagnosis or treatment of UC, or can be used in combination with one or more other UC pANCA target antigens such as those disclosed herein.

Thus, the present invention provides methods of diagnosing UC by obtaining a sample from a subject suspected of having inflammatory bowel disease; contacting the sample with a Bacteroides antigen, or pANCA-reactive fragment thereof, under conditions suitable to form a complex of the Bacteroides antigen, or pANCA-reactive fragment thereof, and antibody to the Bacteroides antigen; and detecting the presence or absence of the complex, where the presence of the complex indicates that the subject has ulcerative colitis. A Bacteroides antigen useful in these methods can be, for example, a *Bacteroides caccae* protein immunoreactive with NANUC-2 and having a molecular weight of about 100 kDa by SDS-PAGE electrophoresis.

The present invention also provides methods of diagnosing a pANCA-positive clinical subtype of ulcerative colitis in a patient with UC. A pANCA-positive clinical subtype of UC can be diagnosed, for example, by obtaining a sample from a patient with UC; contacting the sample with a Bacteroides antigen, or pANCA-reactive fragment thereof, under conditions suitable to form a complex of the Bacteroides antigen, or pANCA-reactive fragment thereof, and antibody to Bacteroides antigen; and detecting the presence or absence of the complex, where the presence of the complex indicates that the patient has the pANCA-positive clinical subtype of UC.

As used herein, the term "Bacteroides antigen" means a microbial pANCA-reactive protein of about 100 kDa that is selectively expressed, at least in part, in Bacteroides. A Bacteroides antigen can be a *Bacteroides caccae* antigen and can be isolated as described in Example VI. For example, a Bacteroides antigen can be a pANCA-reactive protein of about 100 kDa selectively expressed in Bacteroides and containing one or more of the amino acid sequences shown as SEQ ID NOS: 44 through 59 in Table 3. Such a Bacteroides antigen can contain, for example, one of SEQ ID NOS: 42 and 43; and one of SEQ ID NOS: 44 and 45; and one of SEQ ID NOS: 46 to 59.

Also provided herein is a microbial pANCA-reactive antigen of about 75 kDa selectively expressed, at least in part, in several *Bacteroides thetaiotaomicron* strains. Such an isolated microbial pANCA-reactive antigen is shown in Figure 10 (see, also, Example VID).

A sample useful in the methods of the invention can be obtained from any biological fluid having pANCA such as, for example, whole blood, plasma or other bodily fluid or tissue having pANCA, preferably serum. As used herein, the term "patient" means any animal capable of producing pANCA, including, for example, a human, non-human primate, rabbit, rat or mouse. A sample to be assayed according to the methods of the invention can be obtained from any such patient.

As used herein, the term "complex" is synonymous with "immune complex" and means an aggregate of two or more molecules that results from specific binding between an antigen, such as a protein or peptide, and an antibody. For example, a complex can be formed by specific binding of histone H1 to an antibody against histone H1.

As used herein, the term "antibody" means a population of immunoglobulin molecules, which can be polyclonal or monoclonal and of any isotype. As used herein, the term antibody encompasses an immunologically active fragment of an immunoglobulin molecule. Such an immunologically active fragment contains the heavy and light chain variable regions, which make up the portion of the antibody molecule that specifically binds an antigen. For example, an immunologically active fragment of an immunoglobulin molecule known in the art as Fab, Fab' or $F(ab')_2$ is included within the meaning of the term antibody.

As used herein, the term "secondary antibody" means an antibody or combination of antibodies, which binds pANCA of UC. Preferably, a secondary antibody does not compete with histone H1 or another antigen of the

invention for binding to pANCA. A secondary antibody can be an anti-pANCA antibody that binds any epitope of pANCA. A particularly useful secondary antibody is an anti-IgG antibody having specificity for the class determining portion of pANCA. A useful secondary antibody is specific for the species of the ANCA to be detected. For example, if human serum is the sample to be assayed, mouse anti-human IgG can be a useful secondary antibody. A combination of different antibodies, which can be useful in the methods of the invention, also is encompassed within the meaning of the term secondary antibody, provided that at least one antibody of the combination binds pANCA of UC.

As used herein, the term "class determining portion," when used in reference to a secondary antibody, means the heavy chain constant-region sequence of an antibody that determines the isotype, such as IgA, IgD, IgE, IgG or IgM. Thus, a secondary antibody that has specificity for the class determining portion of an IgG molecule, for example, binds IgG in preference to other antibody isotypes.

A secondary antibody useful in the invention can be obtained commercially or by techniques well known in the art. Such an antibody can be a polyclonal or, preferably, monoclonal antibody that binds pANCA. For example, IgG reactive polyclonal antibodies can be prepared using IgG or Fc fragments of IgG as an immunogen to stimulate the production of antibodies in the antisera of an animal such as a rabbit, goat, sheep or rodent, as described in Harlow and Lane, Antibodies: A Laboratory Manual New York: Cold Spring Harbor Laboratory (1988), which is incorporated herein by reference.

A monoclonal antibody also is useful in the practice of the invention. As used herein, a monoclonal antibody refers to a population of antibody molecules that contain only one species of idiotope capable of binding a particular antigen epitope. Methods of producing a monoclonal antibody are well known (see, for example, Harlow and Lane, *supra*, 1988). An immunogen useful in generating a monoclonal antibody that binds pANCA can be, for example, human IgG or a Fc fragment of human IgG, pANCA or a Fab fragment of pANCA. A hybridoma that produces a useful monoclonal antibody can be identified by screening hybridoma supernatants for the presence of antibodies that bind pANCA (Harlow, *supra*, 1988). For example, hybridoma supernatants can be screened using neutrophil and pANCA-positive sera in a radioimmunoassay or enzyme-linked immunosorbent assay. In addition, a monoclonal antibody useful in the invention can be obtained from a number of commercial sources.

The term "detectable secondary antibody" means a secondary antibody, as defined above, that can be detected or measured by analytical methods. Thus, the term secondary antibody includes an antibody labeled directly or indirectly with a detectable marker that can be detected or measured and used in a convenient assay such as an enzyme-linked immunosorbent assay, radioimmunoassay, radial immunodiffusion assay or Western blotting assay, for example. A secondary antibody can be labeled, for example, with an enzyme, radioisotope, fluorochrome or chemiluminescent marker. In addition, a secondary antibody can be rendered detectable using a biotin-avidin linkage such that a detectable marker is associated with the secondary antibody. Labeling of the secondary antibody, however, should not impair binding of

the secondary antibody to pANCA of UC. If desired, a multiple antibody system can be used as the secondary antibody as discussed above. In such a system, at least one of the antibodies is capable of binding pANCA of UC and at least one of the antibodies can be readily detected or measured by analytical methods.

A secondary antibody can be rendered detectable by labeling with an enzyme such as horseradish peroxidase (HRP), alkaline phosphatase (AP), β-galactosidase or urease, for example. A horseradish-peroxidase detection system can be used, for example, with the chromogenic substrate tetramethylbenzidine (TMB), which yields a soluble product in the presence of hydrogen peroxide that is detectable by measuring absorbance at 450 nm. An alkaline phosphatase detection system can be used with the chromogenic substrate *p*-nitrophenyl phosphate, for example, which yields a soluble product readily detectable by measuring absorbance at 405 nm. Similarly, a β-galactosidase detection system can be used with the chromogenic substrate *o*-nitrophenyl-β-D-galactopyranoside (ONPG), which yields a soluble product detectable by measuring absorbance at 410 nm, or a urease detection system can be used with a substrate such as urea-bromocresol purple (Sigma Immunochemicals, St. Louis, MO). A secondary antibody can be linked to an enzyme by methods well known in the art (Harlow and Lane, *supra*, 1988) or can be obtained from a number of commercial sources. For example, goat F(ab')2 anti-human IgG-alkaline phosphatase is a useful detectable secondary antibody that can be purchased from Jackson Immuno-Research (West Grove, PA).

A secondary antibody also can be rendered detectable by labeling with a fluorochrome. Such a

fluorochrome emits light of ultraviolet or visible wavelength after excitation by light or another energy source. DAPI, fluorescein, Hoechst 33258, R-phycocyanin, B-phycoerythrin, R-phycoerythrin, rhodamine, Texas red or lissamine, for example, is a fluorochrome that can be linked to a secondary antibody and used to detect the presence or absence of a complex. A particularly useful fluorochrome is fluorescein or rhodamine. Methods of conjugating and using these and other suitable fluorochromes are described, for example, in Van Vunakis and Langone, Methods in Enzymology, Volume 74, Part C (1991), which is incorporated herein by reference. A secondary antibody linked to a fluorochrome also can be obtained from commercial sources. For example, goat F(ab')2 anti-human IgG-FITC is available from Tago Immunologicals (Burlingame, CA).

A pANCA titer also can be determined using a secondary antibody labeled with a chemiluminescent marker. Such a chemiluminescent secondary antibody is convenient for sensitive, non-radioactive detection of pANCA and can be obtained commercially from various sources such as Amersham Lifesciences, Inc. (Arlington Heights, IL).

A secondary antibody further can be rendered detectable by labeling with a radioisotope. An iodine-125 labeled secondary antibody is a particularly useful detectable secondary antibody (see, for example, Harlow and Lane, *supra*, 1988).

A signal from a detectable secondary antibody can be analyzed, for example, using a spectrophotometer to detect color from a chromogenic substrate; a fluorometer to detect fluorescence in the presence of

light of a certain wavelength; or a radiation counter to detect radiation, such as a gamma counter for detection of iodine-125. For detection of an enzyme-linked secondary antibody, for example, a quantitative analysis of the amount of ANCA can be made using a spectrophotometer such as an EMAX Microplate Reader (Molecular Devices, Menlo Park, CA) in accordance with the manufacturer's instructions. If desired, the assays of the invention can be automated or performed robotically, and the signal from multiple samples can be detected simultaneously.

The assays of the present invention can be forward, reverse or simultaneous as described in U.S. Patent No. 4,376,110, issued March 8, 1983, to David et al., which is incorporated herein by reference. In the forward assay, each reagent is sequentially contacted with histone H1 or another antigen of the invention. If desired, separation of bound from unbound reagent can be performed before the addition of the next reagent. In a reverse assay, all reagents are pre-mixed prior to contacting histone H1 or another antigen of the invention. A modified reverse assay is described in U.S. Patent No. 4,778,751 issued October 18, 1988, to El Shami et al., which is incorporated herein by reference. In a simultaneous assay, all reagents are separately but contemporaneously contacted with histone H1 or another antigen of the invention. As used herein, reagent means any component useful to perform the assays of the present invention, for example, the sample, histone H1 or another antigen of the invention, detectable secondary antibody, washing buffer or other solutions.

Separation steps for the various assay formats described herein, including the removal of unbound

secondary antibody from the complex, can be performed by methods known in the art (Harlow and Lane, *supra*, 1988). For example, washing with a suitable buffer can be followed by filtration, aspiration or magnetic separation. If histone H1, another antigen of the invention, or a pANCA-reactive fragment thereof is immobilized on a particulate support, such as on microparticles, the particulate material can be centrifuged, if desired, followed by removal of wash liquid. If histone H1, another antigen of the invention, or a pANCA-reactive fragment thereof is immobilized on a membrane, filter or well, a vacuum or liquid absorbing apparatus can by applied to the opposite side of the membrane, filter or well to draw the wash liquid away from the complex.

The invention also provides methods of determining susceptibility to UC in an individual by obtaining a sample from the individual; contacting the sample with human histone H1, or pANCA-reactive fragment thereof, under conditions suitable to form a complex of human histone H1, or pANCA-reactive fragment thereof, and antibody to human histone H1; and detecting the presence or absence of the complex, where the presence of the complex indicates that the individual has increased susceptibility to UC. Susceptibility to UC in an individual also can be determined by obtaining a sample from the individual; contacting the sample with purified histone H1 isoform $H1^s$-2, or pANCA-reactive fragment thereof, under conditions suitable to form a complex of purified histone H1 isoform $H1^s$-2, or pANCA-reactive fragment thereof, and antibody to histone H1 isoform $H1^s$-2; and detecting the presence or absence of the complex, where the presence of the complex indicates that the individual has increased susceptibility to UC.

The term "individual," as used herein, means any animal capable of producing pANCA, including a human, non-human primate, rabbit, rat or mouse, provided that the animal does not have ulcerative colitis as defined by the clinical, endoscopic and histopathologic parameters disclosed herein. A sample to be assayed according to the methods of the invention can be obtained from any such individual.

As used herein, the term "susceptibility to UC," when used in reference to an individual, means an inability to resist ulcerative colitis disease-causing factors. As used herein, the term "increased susceptibility to UC," as indicated by the presence of a complex of histone H1 and antibody to histone H1, means an increased inability to resist ulcerative colitis disease-causing factors, as compared with an individual from whom a sample is obtained that does not form a complex when contacted with histone H1, or pANCA-reactive fragment thereof. Similarly, increased susceptibility to UC that is indicated by the presence of a complex of a histone H1-like antigen, a porin antigen or a Bacteroides antigen, and antibody to one of these antigens, means an increased inability to resist ulcerative colitis disease-causing factors, as compared with an individual from whom a sample is obtained that does not form such a complex. Increased susceptibility to UC in an individual does not mean the individual will necessarily develop UC. However, increased susceptibility to UC in an individual is associated with an increased probability of having ulcerative colitis in the future.

The term "pANCA-reactive fragment," as used in reference to histone H1, a histone H1-like antigen, a porin antigen or a Bacteroides antigen, means a peptide

or polypeptide portion of the antigen that has pANCA-reactive activity as defined by the ability to form a complex with pANCA. Thus, the term "pANCA-reactive fragment of histone H1," as used herein, means a peptide or polypeptide that has an amino acid sequence having at least 80% identity to a portion of one of the amino acid sequences shown in Figure 1 and pANCA-reactive activity as defined by the ability to form a complex with pANCA. A pANCA-reactive fragment can have from about three amino acids to about 200 amino acids. Preferably, a pANCA-reactive fragment of one of the UC pANCA antigens disclosed herein has from about five to about fifty amino acids and most preferably from about eight to about twenty amino acids.

Several pANCA-reactive fragments of histone H1 are disclosed herein. As set forth in Examples II and III, peptides SEQ ID NO: 13, SEQ ID NO: 14 and SEQ ID NO: 20 are pANCA-reactive fragments of histone H1, identified by their reactivity with NANUC-1 and NANUC-2. Thus, pANCA-reactive fragments of histone H1 can include, for example, the amino acid sequence of SEQ ID NO: 7, SEQ ID NO: 13, SEQ ID NO: 14 or SEQ ID NO: 20. A pANCA-reactive fragment of histone H1 can have, for example, the amino acid sequence Pro-Lys-Lys-Ala-Lys-Lys-Pro-Ala-Ala-Ala-Thr-Val-Thr-Lys-Lys (SEQ ID NO: 20).

As disclosed herein, a pANCA-reactive fragment of histone H1 can be, for example, SEQ ID NO: 34 (amino acids 160-174 of H1.5); SEQ ID NO: 35 (amino acids 170 to 184 of H1.5) or SEQ ID NO: 36 (amino acids 180-194 of H1.5). As also disclosed herein, a pANCA-reactive fragment of histone H1 can be SEQ ID NO: 37 (amino acids 172 to 184 of H1.5); SEQ ID NO: 38 (amino acids 69 to 184 of H1.5); SEQ ID NO: 39 (amino acids 69 to 171 of H1.5);

SEQ ID NO: 40 (amino acids 69 to 226 of H1.5); or SEQ ID NO: 41 (amino acids 172 to 226 of H1.5), which were identified as pANCA-reactive fragments of histone H1 by their reactivity with NANUC-2 (see Example IV). Reactivity of pANCA antibody with these fragments indicates that a pANCA reactive fragment of histone H1 can have an epitope within or around amino acids 172 to 184 of histone H1.5 (SEQ ID NO: 37). An additional UC pANCA epitope can be localized to amino acids 69 to 171 of histone H1.5, as shown by the reactivity of SEQ ID NO: 39 with NANUC-2.

A pANCA-reactive fragment of an antigen disclosed herein can be identified by the ability to form a complex with pANCA. For example, a pANCA-reactive fragment of histone H1, a histone H1-like antigen, a porin antigen or a Bacteroides antigen can be identified by its ability to form a complex with pANCA when contacted with pANCA-positive UC sera. Assays for the formation of an antigen-pANCA complex using pANCA-positive sera are well known in the art. For example, an enzyme-linked immunosorbent assay (ELISA) as described in Saxon et al., J. Allergy Clin. Immunol. 86:202-210 (1990), which is incorporated herein by reference, is particularly useful in identifying a pANCA-reactive fragment that forms a complex with pANCA.

A pANCA-reactive fragment of histone H1, a histone H1-like antigen, a porin antigen or a Bacteroides antigen further can be identified by its ability to form a complex with a representative UC pANCA monoclonal antibody, such as NANUC-2. The sequences of the NANUC-2 heavy and light chains are provided herein, and assays for determining binding to NANUC-2 are described in Examples IA and IB. An ELISA assay, for example, is

particularly useful in identifying a pANCA-reactive fragment. Example II describes identification of the pANCA-reactive fragments of histone H1 SEQ ID NO: 13 and SEQ ID NO: 14, and Example II describes identification of the pANCA-reactive fragment SEQ ID NO: 20 using ELISA analysis. One skilled in the art understands that a pANCA-reactive fragment of a histone H1-like antigen, a porin antigen or a Bacteroides antigen can be similarly identified.

The histone H1, histone H1-like, porin and Bacteroides antigens of the invention also can be useful in treating UC. The present invention provides, for example, methods of inducing tolerance in a pANCA-positive patient with UC by administering an effective dose of histone H1, or tolerogenic fragment thereof, to the pANCA-positive patient with UC. A tolerogenic fragment of histone H1 useful in the methods of the invention can include, for example, the amino acid sequence of SEQ ID NO: 7, SEQ ID NO: 13, SEQ ID NO: 14 or SEQ ID NO: 20. A particularly useful tolerogenic fragment of histone H1 includes the amino acid sequence of SEQ ID NO: 20. A tolerogenic fragment of histone H1 can be, for example, a 15-mer having amino acid sequence Pro-Lys-Lys-Ala-Lys-Lys-Pro-Ala-Ala-Ala-Thr-Val-Thr-Lys-Lys (SEQ ID NO: 20).

The invention also provides methods of inducing tolerance in a pANCA-positive patient with UC by administering to the patient an effective dose of a histone-like H1 antigen, or tolerogenic fragment thereof. Such a histone H1-like antigen can be, for example, a protein immunoreactive with NANUC-2 and having an amino acid sequence having at least 65% amino acid identity with SEQ ID NO: 27.

The invention also provides methods of inducing tolerance in a pANCA-positive patient with UC by administering to the patient an effective dose of a porin antigen, or tolerogenic fragment thereof. Such a porin antigen can be, for example, a protein immunoreactive with NANUC-2 and having at least 65% amino acid identity with SEQ ID NO: 28, SEQ ID NO: 29 or SEQ ID NO: 30.

The invention also provides methods of inducing tolerance in a pANCA-positive patient with UC by administering to the patient an effective dose of a Bacteroides antigen, or tolerogenic fragment thereof. Such a Bacteroides antigen can be, for example, a *Bacteroides caccae* protein immunoreactive with NANUC-2 and having a molecular weight of about 100 kDa by SDS-PAGE electrophoresis.

The term "tolerogenic fragment," as used in reference to histone H1, a histone H1-like antigen, a porin antigen or a Bacteroides antigen, means a peptide or polypeptide portion of the antigen that has tolerogenic activity as defined by its ability either alone, or in combination with another molecule, to produce a decreased immunological response. For example, the term "tolerogenic fragment of histone H1," as used herein, means a peptide or polypeptide which has an amino acid sequence having at least 80% identity to a portion of one of the amino acid sequences shown in Figure 1 and tolerogenic activity as defined by its ability either alone, or in combination with another molecule, to produce a decreased immunological response. A tolerogenic fragment of histone H1, a histone H1-like antigen, a porin antigen or a Bacteroides antigen has from about three amino acids to about 200 amino acids. Preferably, a tolerogenic fragment of histone H1, a

histone H1-like antigen, a porin antigen or a Bacteroides antigen has from about five to about fifty amino acids and most preferably from about eight to about twenty amino acids.

A particularly useful tolerogenic fragment of histone H1, a histone H1-like antigen, a porin antigen or a Bacteroides antigen can be a cryptic T-cell determinant that normally is not the target of T-cell recognition due to inefficient processing and antigen presentation (see, for example, Sercarz et al., Ann. Rev. Immunol. 11:729 (1993), which is incorporated herein by reference). Without wishing to be bound by the following, ulcerative colitis can be associated with an immune response to histone H1 in disease tissue due to expression of a normally cryptic histone H1 T-cell determinant in an immunogenic form in disease target tissues but not in other tissues.

As disclosed herein, a variety of cell types have substantial amounts of NANUC-2 reactive histone H1, as assayed by Western analysis which involves denatured histone H1. In particular, all cell types assayed by Western analysis, including both hematopoietic and non-hematopoietic cells such as neutrophils, lymphocytes, Molt-4, HL60 promyelocytic leukemia and COS cells, have NANUC-2 reactive histone H1. However, as disclosed herein, only neutrophils and HL60 cells are reactive with NANUC-2 or UC sera when cells are methanol-fixed and assayed by an immunohistochemical analysis involving native protein. These results indicate that native, but not denatured, histone H1 can have different immunoaccessibility properties in neutrophilic and non-neutrophilic cell types.

The cell-type specific immunoaccessibility of histone H1 is supported by polyclonal rabbit anti-histone H1 antisera directed against an N-terminal 35 amino acid fragment of human histone H1 isoform $H1^{s}$-2 (SEQ ID NO: 7). The antiserum against SEQ ID NO: 7 recognizes all histone H1 isoforms and has nearly identical reactivity profiles as NANUC-2 using Western analysis. Furthermore, the antisera against SEQ ID NO: 7, like pANCA-positive sera from UC patients, stains methanol-fixed neutrophils in a perinuclear distribution but not non-neutrophilic cells such as eosinophils or lymphocytes.

A tolerogenic fragment of histone H1, a histone H1-like antigen, a porin antigen or a Bacteroides antigen can be identified using a variety of assays, including *in vitro* assays such as T-cell proliferation or cytokine secretion assays and *in vivo* assays such as the induction of tolerance in murine models of ulcerative colitis. T-cell proliferation assays, for example, are well recognized in the art as predictive of tolerogenic activity (see, for example, Miyahara et al., Immunol. 86:110-115 (1995) or Lundin et al, J. Exp. Med. 178:187-196 (1993), each of which is incorporated herein by reference). A T-cell proliferation assay can be performed by culturing T-cells with irradiated antigen-presenting cells, such as normal spleen cells, in microtiter wells for 3 days with varying concentrations of a fragment of histone H1, a histone H1-like antigen, a porin antigen or a Bacteroides antigen to be assayed; adding ^{3}H-thymidine; and measuring incorporation of ^{3}H-thymidine into DNA. In such an assay, a fragment of histone H1, a histone H1-like antigen, a porin antigen or a Bacteroides antigen can be tested for activity, for example, at concentrations of 20 µg/ml and 40 µg/ml.

A tolerogenic fragment of histone H1, a histone H1-like antigen, a porin antigen or a Bacteroides antigen also can be identified using a T-cell cytokine secretion assay as is well known in the art. For example, T cells can be cultured with irradiated antigen-presenting cells in microtiter wells with varying concentrations of the fragment of interest and, after three days, the culture supernatants can be assayed for IL-2, IL-4 or IFN-γ as described in Czerinsky et al., Immunol. Rev. 119:5-22 (1991), which is incorporated herein by reference.

Primary T-cells for use in a T-cell proliferation assay or cytokine secretion assay, for example, can be isolated from lamina propria or peripheral blood. In addition, a convenient source of T-cells for use in an *in vitro* assay for tolerogenic activity can be a T-cell line established from an ulcerative colitis patient, murine model of ulcerative colitis or a healthy animal immunized with histone H1. A preferred source of T-cells for use in identifying a tolerogenic fragment of histone H1, a histone H1-like antigen, a porin antigen or a Bacteroides antigen is an ulcerative colitis patient.

A T-cell line can be established from a patient with UC, for example, by culturing T lymphocytes with about 1 µg/ml histone H1, which is prepared, for example, from human bone marrow as described in Example I, in the presence of low concentrations of growth-supporting IL-2 (about 10 µg/ml). A T-cell line can be established by culturing T lymphocytes with antigen-presenting cells and feeding the cells on an alternating four to five day cycle with either IL-2 and histone H1 or IL-2 alone as described in Nanda et al., J. Exp. Med. 176:297-302 (1992), which is incorporated herein by reference. A

cell line that develops into a reliably proliferating cell line dependent on the presence of histone H1 is particularly useful in identifying tolerogenic fragments of histone H1. The establishment of T-cell lines from small intestinal mucosa is described, for example, in Lundin et al., *supra*, 1993. T cell lines dependent upon the presence of a histone H1-like antigen, a porin antigen or a Bacteroides antigen can be prepared similarly and used to identify tolerogenic fragments of a histone H1-like antigen, a porin antigen or a Bacteroides antigen, respectively.

A tolerogenic fragment of histone H1, a histone H1-like antigen, a porin antigen or a Bacteroides antigen can also be identified by its ability to induce tolerance *in vivo*, as indicated by a decreased immunological response, which can be a decreased T-cell response, such as a decreased proliferative response or cytokine secretion response as described above, or a decreased antibody titer to the antigen. A neonatal or adult mouse can be tolerized with a fragment of histone H1, for example, and a T-cell response or anti-histone H1 antibody titer can be assayed after challenging by immunization. For example, a neonatal mouse can be tolerized within 48 hours of birth by intraperitoneal administration of about 100 µg of a fragment of histone H1 emulsified with incomplete Freund's adjuvant and subsequently immunized with histone H1 at about 8 weeks of age (see, for example, Miyahara, *supra*, 1995). An adult mouse can be tolerized intravenously with about 0.33 mg of a fragment of histone H1, administered daily for three days (total dose 1 mg), and immunized one week later with histone H1. A decreased T-cell response, such as decreased proliferation or cytokine secretion, which indicates tolerogenic activity, can be measured using

T-cells harvested 10 days after immunization. In addition, a decreased anti-histone H1 antibody titer, which also indicates tolerogenic activity, can be assayed using blood harvested 4-8 weeks after immunization. Methods for assaying a T-cell response or anti-histone H1 antibody titer are described above and are well known in the art. Neonatal or adult mice can be tolerized similarly with a histone H1-like antigen, a porin antigen, or a Bacteroides antigen and used to identify tolerogenic fragments of the histone H1-like antigen, the porin antigen or the Bacteroides antigen, respectively.

A tolerogenic fragment of histone H1, a histone H1-like antigen, a porin antigen or a Bacteroides antigen also can be identified using a murine model of ulcerative colitis. Neonatal or adult mice having ulcerative colitis-like disease can be tolerized with a fragment of histone H1, a histone H1-like antigen, a porin antigen or a Bacteroides antigen as described above, and the T-cell response or anti-histone antibody titer assayed. A decreased T-cell response or decreased antibody titer to the antigen indicates a decreased immunological response and, thus, serves to identify a tolerogenic fragment of histone H1, the histone H1-like antigen, porin antigen or Bacteroides antigen. In addition, a tolerogenic fragment of histone H1, a histone H1-like antigen, a porin antigen or a Bacteroides antigen can be identified by the ability to reduce the frequency, time of onset or severity of colitis in a murine model of UC.

Several well-accepted murine models of ulcerative colitis are useful in identifying a tolerogenic fragment of histone H1 or another UC pANCA antigen disclosed herein. For example, mice deficient in IL-2 as described in Sadlack et al., Cell 75:253-261

(1993), which is incorporated herein by reference, and mice deficient in IL-10 as described in Kühn et al., Cell 75:263-274 (1993), which is incorporated herein by reference, have ulcerative-colitis like disease and are useful in identifying a tolerogenic fragment of histone H1, a histone H1-like antigen, a porin antigen or a Bacteroides antigen. Furthermore, mice with mutations in T cell receptor (TCR) α, TCR β, TCR β x δ, or the class II major histocompatiblility complex (MHC) as described in Mombaerts et al., Cell 75:275-282 (1993), which is incorporated herein by reference, develop inflammatory bowel disease that resembles ulcerative colitis and, thus, are useful in identifying a tolerogenic fragment of histone H1, a histone H1-like antigen, a porin antigen or a Bacteroides antigen. Similarly, a fragment can be assayed for tolerogenic activity in a SCID mouse reconstituted with CD45RB CD4+ T-cells, which is a well-accepted model of human ulcerative colitis, as described in Powrie et al., Immunity 1:553-562 (1994), which is incorporated herein by reference. Thus, a tolerogenic fragment of histone H1, a histone H1-like antigen, a porin antigen or a Bacteroides antigen can be readily identified by an *in vitro* or *in vivo* assay disclosed herein or by another assay well known in the art.

A pANCA-reactive or tolerogenic fragment can be identified by screening, for example, fragments of the antigen produced by chemical or proteolytic cleavage. A fragment prepared from histone H1 purified from a target tissue such as intestinal mucosa can be particularly useful since such a fragment can have a post-translational modification that contributes to pANCA-reactive activity or tolerogenic activity. Methods for chemical and proteolytic cleavage and for

purification of the resultant protein fragments are well known in the art (see, for example, Deutscher, Methods in Enzymology, Vol. 182, "Guide to Protein Purification," San Diego: Academic Press, Inc. (1990), which is incorporated herein by reference). For example, a chemical such as cyanogen bromide or a protease such as trypsin, chymotrypsin, V8 protease, endoproteinase Lys-C, endoproteinase Arg-C or endoproteinase Asp-N can be used to produce convenient fragments of histone H1, a histone H1-like antigen, a porin antigen or a Bacteroides antigen that can be screened for pANCA-reactive activity or tolerogenic activity using one of the assays disclosed herein.

A pANCA-reactive or tolerogenic fragment of histone H1, a histone H1-like antigen, a porin antigen or a Bacteroides antigen also can be identified by screening a large collection, or library, of random peptides or peptides of interest for pANCA-reactive activity or tolerogenic activity. Peptide libraries include, for example, tagged chemical libraries comprising peptides and peptidomimetic molecules. Peptide libraries also comprise those generated by phage display technology. Phage display technology includes the expression of peptide molecules on the surface of phage as well as other methodologies by which a protein ligand is or can be associated with the nucleic acid which encodes it. Methods for production of phage display libraries, including vectors and methods of diversifying the population of peptides which are expressed, are well known in the art (see, for example, Smith and Scott, Methods Enzymol. 217:228-257 (1993); Scott and Smith, Science 249:386-390 (1990); and Huse, WO 91/07141 and WO 91/07149, each of which is incorporated herein by reference). These or other well known methods can be

used to produce a phage display library which can be screened, for example, with one of the disclosed assays for pANCA-reactive activity or tolerogenic activity. If desired, a population of peptides can be assayed for activity *en masse*. For example, to identify a pANCA-reactive fragment of histone H1, a histone H1-like antigen, a porin antigen or a Bacteroides antigen, a population of peptides can be assayed for the ability to form a complex with NANUC-2; the active population can be subdivided and the assay repeated in order to isolate a pANCA-reactive fragment from the population.

In addition, a peptide library can be a panel of peptides spanning the entire sequence of an antigen of interest. For example, a panel of about 75 individual 15-mer peptides spanning the sequence of human histone H1 isoform $H1^s$-2 (SEQ ID NO: 2) can be synthesized, each overlapping by three residue shifts using the Mimotope cleavable pin technology (Cambridge Research Biochemicals, Wilmington, DE), as described by Geysen et al., Science 235:1184 (1987), which is incorporated herein by reference. A panel of peptides spanning the sequence of any of the histone H1 isoforms such as those shown in Figure 1 or another UC pANCA antigen disclosed herein can be generated similarly, and the panel screened for pANCA-reactive activity or tolerogenic activity using one of the assays described above (see, for example, Miyahara et al., *supra*, 1995, which is incorporated herein by reference).

A library of peptides to be screened also can be made up of peptides of interest, such as a population of peptides related in amino acid sequence to SEQ ID NO: 7 or SEQ ID NO: 20 but having one or more amino acids that differ from SEQ ID NO: 7 or SEQ ID NO: 20. For

identifying a tolerogenic fragment of histone H1, peptides of interest also can be peptides derived from a histone H1 sequence that have appropriate HLA-DR binding motifs as described, for example, in Sette et al., J. Immunol. 151:3163-3170 (1993), which is incorporated herein by reference. A particularly useful population of peptides is a population having a HLA-DR2 binding motif (Yang et al., *supra*, 1993). If desired, peptides of interest can be selected for HLA-DR binding activity as described in Sette et al., *supra*, 1993, prior to screening for tolerogenic activity.

As used herein, the term "fragment" means a peptide, polypeptide or compound containing naturally occurring amino acids, non-naturally occurring amino acids or chemically modified amino acids. A pANCA-reactive or tolerogenic fragment of histone H1, a histone H1-like antigen, a porin antigen or a Bacteroides antigen also can be a peptide mimetic, which is a non-amino acid chemical structure that mimics the structure of a peptide having an amino acid sequence, provided that the peptidomimetic retains pANCA-reactive activity or tolerogenic activity, as defined herein. Such a mimetic generally is characterized as exhibiting similar physical characteristics such as size, charge or hydrophobicity in the same spatial arrangement found in its peptide counterpart. A specific example of a peptide mimetic is a compound in which the amide bond between one or more of the amino acids is replaced, for example, by a carbon-carbon bond or other bond well known in the art (see, for example, Sawyer, Peptide Based Drug Design, ACS, Washington (1995), which is incorporated herein by reference).

As used herein, the term "amino acid" refers to one of the twenty naturally occurring amino acids, including, unless stated otherwise, L-amino acids and D-amino acids. The term amino acid also refers to compounds such as chemically modified amino acids including amino acid analogs, naturally occurring amino acids that are not usually incorporated into proteins such as norleucine, and chemically synthesized compounds having properties known in the art to be characteristic of an amino acid, provided that the compound can be substituted within a peptide such that it retains pANCA-reactive activity or tolerogenic activity. Examples of amino acids and amino acids analogs are listed in Gross and Meienhofer, The Peptides: Analysis, Synthesis, Biology, Academic Press, Inc., New York (1983), which is incorporated herein by reference. An amino acid also can be an amino acid mimetic, which is a structure that exhibits substantially the same spatial arrangement of functional groups as an amino acid but does not necessarily have both the α-amino and α-carboxyl groups characteristic of an amino acid.

A pANCA-reactive or tolerogenic fragment of histone H1, a histone H1-like antigen, a porin antigen or a Bacteroides antigen useful in the invention can be produced or synthesized using methods well known in the art. Such methods include recombinant DNA methods and chemical synthesis methods for production of a peptide. Recombinant methods of producing a peptide through expression of a nucleic acid sequence encoding the peptide in a suitable host cell are well known in the art and are described, for example, in Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd Ed, Vols 1 to 3, Cold Spring Harbor Laboratory Press, New York (1989), which is incorporated herein by reference.

Nucleic acids encoding histone H1 are available to one skilled in the art as described in Eick et al., Eur. J. Cell. Biol. 49:110-115 (1989); Albig et al., Genomics 10:940-948 (1991); Carozzi et al., Science 224:1115-1117 (1984); La Bella et al., J. Biol. Chem. 263:2115-2118 (1988); Cole et al., Gene 89:265-269 (1990); Cheng et al., Proc. Natl. Acad. Sci. USA 86:7002-7006 (1989); Yan et al., J. Biol. Chem. 262:17118-17125 (1987); and Brown and Sitman, J. Biol. Chem. 268:713-718 (1993), each of which is incorporated herein by reference.

A pANCA-reactive or tolerogenic fragment of histone H1, a histone H1-like antigen, a porin antigen or a Bacteroides antigen useful in the invention also can be produced by chemical synthesis, for example, by the solid phase peptide synthesis method of Merrifield et al., J. Am. Chem. Soc. 85:2149 (1964), which is incorporated herein by reference. Standard solution methods well known in the art also can be used to synthesize a pANCA-reactive or tolerogenic fragment useful in the invention (see, for example, Bodanszky, Principles of Peptide Synthesis, Springer-Verlag, Berlin (1984) and Bodanszky, Peptide Chemistry, Springer-Verlag, Berlin (1993), each of which is incorporated herein by reference). A newly synthesized peptide can be purified, for example, by high performance liquid chromatography (HPLC), and can be characterized using, for example, mass spectrometry or amino acid sequence analysis.

It is understood that limited modifications can be made to histone H1, a histone H1-like antigen, a porin antigen or a Bacteroides antigen without destroying its biological function. Similarly, limited modifications can be made to a pANCA-reactive fragment of an antigen disclosed herein or a tolerogenic fragment of an antigen

disclosed herein without destroying its pANCA-reactive activity or tolerogenic activity. A modification of an antigen disclosed herein that does not destroy pANCA-reactive activity or a modification of an antigen disclosed herein that does not destroy tolerogenic activity is within the definition of such antigen. Similarly, a modification of a pANCA-reactive fragment of an antigen disclosed herein that does not destroy its ability to form a complex with pANCA is within the definition of a pANCA-reactive fragment of such antigen. Furthermore, a modification of a tolerogenic fragment of an antigen disclosed herein that does not destroy its ability to produce a decreased immunological response is within the definition of a tolerogenic fragment of such antigen. A modification can be, for example, an addition, deletion, or substitution of amino acid residues; substitution of a compound that mimics amino acid structure or function; or addition of chemical moieties such as amino or acetyl groups. The activity of a modified antigen disclosed herein or a modified fragment of such antigen can be assayed, for example, using one of the assays for pANCA-reactive or tolerogenic activity disclosed herein.

A particularly useful modification of an antigen disclosed herein or a pANCA-reactive or tolerogenic fragment of such antigen is a modification that confers, for example, increased stability. Incorporation of one or more D-amino acids is a modification useful in increasing stability of a protein or protein fragment. Similarly, deletion or substitution of lysine can increase stability by protecting against degradation. For example, such a substitution can increase stability and, thus, bioavailability of an antigen disclosed herein or a tolerogenic fragment of

such antigen, provided that the substitution does not affect tolerogenic activity.

As used herein, the term "effective dose" means the amount of histone H1, a histone H1-like antigen, a porin antigen or a Bacteroides antigen, or a tolerogenic fragment thereof, useful for inducing tolerance in a pANCA-positive patient with UC. For induction of oral tolerance, for example, multiple smaller oral doses can be administered or a large dose can be administered. Such doses can be extrapolated, for example, from the induction of tolerance in animal models (see, for example, Trentham et al., Science 261:1727-1730 (1993), which is incorporated herein by reference).

The present invention also provides tolerogenic compositions that contain a UC pANCA antigen and are useful in inducing tolerance in a patient with UC. In particular, the invention provides a composition of histone H1, or fragment thereof, and a tolerogizing molecule. A composition of the invention can contain a fragment of histone H1 including the amino acid sequence of SEQ ID NO: 7, SEQ ID NO: 13, SEQ ID NO: 14 or SEQ ID NO: 20 combined with a tolerogizing molecule. A composition of the invention also can contain a fragment of histone H1 having the amino acid sequence of SEQ ID NO: 20 combined with a tolerogizing molecule.

The present invention also provides a composition of histone H1-like antigen, or tolerogenic fragment thereof, combined with a tolerogizing molecule. In such a composition, a histone H1-like antigen can be, for example, a protein having an amino acid sequence having at least 65% amino acid identity with SEQ ID NO: 27.

In addition, there is provided a porin antigen, or tolerogenic fragment thereof, combined with a tolerogizing molecule. In a composition of the invention, a porin antigen can be, for example, a protein having at least 65% amino acid identity with SEQ ID NO: 28, SEQ ID NO: 29 or SEQ ID NO: 30.

Also provided herein is a composition containing a Bacteroides antigen, or a tolerogenic fragment thereof, combined with a tolerogizing molecule. Such a Bacteroides antigen can be, for example, a *Bacteroides caccae* protein having a molecular weight of about 100 kDa by SDS-PAGE electrophoresis.

Various molecules are known in the art to cause, promote or enhance tolerance. See, for example, U.S. Patent No. 5,268,454, and citations therein, which are incorporated herein by reference. As used herein, the term "tolerogizing molecule" means a molecule, compound or polymer that causes, promotes or enhances tolerogenic activity when combined with histone H1, a histone H1-like antigen, a porin antigen or a Bacteroides antigen, or fragment thereof. A tolerogizing molecule can be, for example, conjugated to histone H1 or another UC pANCA antigen. Such tolerogizing molecules include, for example, polyethylene glycol and are well known in the art (see, for example, U.S. Patent No. 5,268,454, *supra*).

An effective dose of histone H1, a histone H1-like antigen, a porin antigen, or a Bacteroides antigen or a tolerogenic fragment thereof for inducing tolerance can be administered by methods well known in the art. Oral tolerance is well-recognized in the art as a method of treating autoimmune disease (see, for

example, Weiner, Hospital Practice, pp. 53-58 (Sept. 15, 1995), which is incorporated herein by reference). For example, orally administered autoantigens suppress several experimental autoimmune models in a disease- and antigen-specific fashion; the diseases include experimental autoimmune encephalomyelitis, uveitis, and myasthenia, collagen- and adjuvant-induced arthritis, and diabetes in the NOD mouse (see, for example, Weiner et al., Ann. Rev. Immunol. 12:809-837 (1994), which is incorporated herein by reference). Furthermore, clinical trials of oral tolerance have produced positive results in treating multiple sclerosis, rheumatoid arthritis and uveitis. In addition, parenteral administration of histone H1, another antigen of the invention, or a tolerogenic fragment thereof, can be used to induce tolerance. Subcutaneous injection, for example, can be used to deliver histone H1, a histone H1-like antigen, a porin antigen or a Bacteroides antigen, or a tolerogenic fragment thereof, to a pANCA-positive patient with UC (Johnson, Ann. Neurology 36(suppl.):S115-S117 (1994), which is incorporated herein by reference).

The invention also provides methods of preventing UC in an individual by administering an effective dose of histone H1, a histone H1-like antigen, a porin antigen or a Bacteroides antigen, or a tolerogenic fragment thereof, to the individual. The methods of the invention are particularly useful for preventing UC in an individual having increased susceptibility to UC. Such methods can be particularly useful for preventing UC when an effective dose of the antigen or tolerogenic fragment is administered to a newborn individual.

Prior to this time, a connection between specific enteric bacteria and UC has not been made although bacteria have been shown to play a role in the pathogenesis of other enteric diseases. For example, *H. pylori* has been implicated in the pathogenesis of peptic ulcer disease. Antibiotics against *H. pylori* have been shown to effectively treat this disease (see, for example, Sontag, Am. J. Gastroenterol. 92:1255-1261 (1997); and Pipkin et al., Helicobactor. 2:159-171 (1997), each of which is incorporated herein by reference). As disclosed herein, several colonic bacteria harbored in UC patients, including bacteria from the genera of Mycobacteria, Escherichia and Bacteroides, express antigens reactive with the pANCA autoantibody, which is present in the majority of patients with UC (see Examples V and VI). These results indicate that an agent directed against one or more of such pANCA-reactive bacteria can be useful in reducing the number of colonic bacteria, thereby diminishing the immune stimulus contributing to UC and ameliorating the symptoms of the disease.

Thus, the invention provides a method of identifying an agent useful for treating UC by obtaining a sample of enteric bacteria from a patient with UC; isolating from the sample a bacterial species that expresses a pANCA-reactive antigen; contacting the bacterial species with an agent; and assaying for the reduced growth or viability of the bacterial species, where the reduced growth or viability of the bacterial species indicates that the agent is an agent useful for treating UC. The enteric bacterial species that expresses a pANCA-reactive antigen can be, for example, a member of the genera of Mycobacteria, Escherichia or Bacteroides. An enteric Mycobacteria that expresses a

pANCA-reactive antigen can be, for example, *Mycobacterium tuberculosis, Mycobacterium bovis, Mycobacterium smegmatis [1-2c], Mycobacterium avium* and *Mycobacterium avium paratuberculosis*. An enteric Escherichia that expresses a pANCA-reactive antigen can be, for example, *Escherichia coli*. An enteric Bacteroides that expresses a pANCA-reactive antigen can be, for example, *Bacteroides caccae* or *Bacteroides thetaiotaomicron*. One skilled in the art understands that the bacterial species which is contacted with an agent in the methods of the invention can be a single bacterial species or can be a mixture of two or more bacterial species that express a pANCA-reactive antigen.

As used herein, the term "agent" means a biological or chemical compound such as a simple or complex organic molecule, a peptide, a protein, an antibody, a lipid or an oligonucleotide.

As used herein, the term "agent useful for treating UC" means an agent that can reduce the viability or growth of a bacterial species that expresses a pANCA-reactive antigen. Thus, an agent useful for treating UC is an agent that functions as a bacteristat or bactericide against a bacterial species that expresses a pANCA-reactive antigen. An agent useful for treating UC can be a bacterial antibiotic, which is a molecule that is produced by a microorganism or a plant, or a close chemical derivative of such a molecule, that can reduce the growth or viability of a bacterial species that expresses a pANCA-reactive antigen. An agent useful for treating UC can function by a variety of mechanisms, for example, by inhibiting bacterial protein synthesis, inhibiting bacterial DNA synthesis, inhibiting the synthesis of a bacterial cell wall or inhibiting

synthesis of an essential nutrient of a bacterial species that expresses a pANCA-reactive antigen. Such an agent can selectively reduce the viability or growth of a particular bacterial species that expresses a pANCA-reactive antigen. An agent useful for treating UC also can have activity in reducing the growth or viability of a broad spectrum of bacteria such as a genus of bacteria. One skilled in the art understands that, preferably, an agent useful for treating UC reduces the growth or viability of a bacterial species that expresses a pANCA-reactive antigen without significantly altering the growth or viability of mammalian cells, especially human cells.

The following examples are intended to illustrate but not limit the present invention.

EXAMPLE I

IDENTIFICATION OF THE ULCERATIVE COLITIS pANCA TARGET ANTIGEN

This example demonstrates that representative UC pANCA monoclonal antibodies bind histone H1 specifically.

A. Histone H1 is an ulcerative colitis pANCA target antigen

Representative UC pANCA monoclonal antibodies, designated NANUC-1 and NANUC-2, were isolated from a UC lamina propria lymphocyte phage display IgG library and use to screen human neutrophil. Western analysis demonstrated specific binding of NANUC-2 to a nuclear protein doublet of 32-33 kDa. Purification by subcellular fractionation and preparative gel

electrophoresis followed by protein microsequencing identified the NANUC-2 reactive antigen as histone H1.

Western analysis showed reactivity with lysine-rich calf thymus histone. In addition, histones purified from human neutrophil were fractionated into a perchloric acid insoluble fraction (containing core histones) and a perchloric acid soluble fraction (containing histone H1). As shown in Figure 2, NANUC-2 reacted with the perchloric acid soluble histone fraction, indicating that histone H1 is an ulcerative colitis pANCA target antigen. Purified core histones (H2A, H2B, H3 and H4) were minimally reactive with NANUC-2. In addition, purified human histone H1 isoforms $H1^{s}$-1, $H1^{s}$-2, $H1^{s}$-3, $H1^{s}$-4 and $H1^{\circ}$ were analyzed by immunoblot analysis, and NANUC-2 was reactive with each of the isoforms including $H1^{\circ}$.

Histone H1 was purified according to the methods described in Prescott, Methods in Cell Biology, Vol XVI, "Chromatin and Chromosomal Protein Research" (New York, NY: Academic Press (1977)), which is incorporated herein by reference, as follows. Purified bone marrow was obtained and red blood cell lysed prior to freezing. The bone marrow, which contained lymphocytes and granulocytes, was thawed rapidly and washed with phosphate-buffered saline (PBS). The cells were extracted with four cell volumes of 200 mM H_2SO_4/40mM $NaHSO_3$ in the presence of protease inhibitors, sonicated for 60 seconds on ice, and incubated on ice for one hour with occasional vortexing. Nuclei and cell debris were pelleted at 2500 rpm (Beckman JA-6) at 4°C for 20 minutes, the supernatant transferred to a new tube, and core histones (H2A, H2A, H3 and H4) precipitated at -20°C overnight by the addition of three to four volumes of 95%

ethanol. The histone pellet was washed with 70% ethanol, dried and resuspended in 3 ml 40mM $NaHSO_3$ with protease inhibitors.

Histone H1 was selectively extracted from core histones by addition of 70% perchloric acid (to a final concentration of 5%) followed by incubation on a rotating wheel at 4°C for 1 hour. Core histones were pelleted at 2500 rpm at 4°C for 20 minutes. Histone H1 was precipitated from the supernatant for 2 hours at -20°C with 10 ml of acidified acetone (10 ml acetone + 77 µl concentrated hydrochloric acid). Histone H1 was centrifuged as above, and the pellet washed with a solution of 3.5 ml acetone/1ml 1M HCl to remove high-mobility group (HMG) proteins. The core histones and histone H1 pellets each were washed separately three times with 5 ml 95% ethanol and dried. Protein purity was established by polyacrylamide gel electrophoresis and Coomassie blue staining.

Western analysis was performed as follows. Cells were lysed in 10mM HEPES/1.5mM $MgCl_2$/10mM KCl pH7.9 in the presence of protease inhibitors and sheared with a 20G needle. Lysis was monitored by trypan blue exclusion. Nuclei were pelleted and resuspended in extraction buffer, and the cell fractions electrophoresed on a 12% polyacrylamide gel under non-reducing conditions. Proteins were transferred to nitrocellulose membranes, and the transfer verified by Ponceau S red staining (SIGMA, St. Louis, MO). Membranes were blocked with 5% milk in 0.1% Tween-20/PBS for 1 hour. Primary and secondary antibody incubations were for 1 hour in 1% milk in 0.1% Tween-20/PBS. The primary antibodies, NANUC-1, NANUC-2, and anti-tetanus toxoid Fabs were used at a concentration of 0.1 to 1.0 µg/ml. The secondary

antibody was goat anti-human Fab-alkaline phosphatase or goat anti-human kappa-biotin used at a dilution of 1 to 1000 or 1 to 2000, respectively. Alkaline phosphatase labeled antibodies were detected with BCIP-NBT (SIGMA). Biotinylated antibodies were detected with SA-HRP (Amersham Lifesciences, Inc., Arlington Heights, IL) and enhanced chemiluminescence.

B. Reactivity of NANUC-2 with histone H1 using ELISA analysis

Microtiter plates coated with neutrophil, total histone or purified calf thymus histone H1 were used for ELISA analysis as described below. The reactivity of NANUC-1, NANUC-2 and negative control anti-tetanus toxoid antibody was tested against human PMN (neutrophil), total histone, calf thymus histone H1, or tetanus toxoid antigen. As shown in Figure 3, the ELISA assays demonstrated that NANUC-1 and NANUC-2 react with human neutrophil. However, NANUC-2, but not NANUC-1 or anti-tetanus toxoid antibody, was reactive with total calf thymus histone (histone H1 and core histones) and calf thymus histone H1 (SIGMA).

ELISA assays were performed as follows. For detection of total Fab immunoglobulin, microtiter plates (Costar 3069, Cambridge, MA) were coated overnight at 4°C with 500ng/well antigen in bicarbonate pH 9.6 coating buffer. Wells were blocked with 0.25% BSA/PBS for 1 hour, incubated with rFabs diluted in 0.25% BSA/PBS for 1 hour, and washed five times with 0.5% Tween-20/PBS at room temperature. Plates were subsequently incubated with a 1 to 1000 dilution of alkaline phosphatase-labeled goat anti-human Fab (Pierce, Rockford, IL) for 1 hour; washed five times in 0.5% Tween-20/PBS; washed three

times with Tris-NaCl (50 mM Tris 150 mM NaCl pH 7.5); and developed with 5 mg/ml *p*-nitrophenyl phosphate (SIGMA) in 10% diethanolamine/1 mM $MgCl_2$ pH 9.8. The absorbance of each sample was measured at 405 nm using a Biorad ELISA reader (Richmond, CA). Neutrophil samples were prepared as described in Saxon et al., *supra*, 1990; total calf thymus histone and calf thymus histone H1 were obtained from SIGMA.

C. Reactivity of NANUC-2 with subcellular fractions

Subcellular fractionation of human neutrophils demonstrated that NANUC-2 is almost exclusively reactive with a nuclear protein doublet of 32-33kDa apparent molecular weight (see Figure 4). The NANUC-2 reactive doublet was present in the nuclear fraction of neutrophils and represents histone H1 subtypes $H1^s$-1, $H1^s$-2, $H1^s$-3, $H1^s$-4 and H1° as determined by apparent size on SDS-PAGE following Western blot detection. Additional lower molecular weight proteins, which have slight reactivity with NANUC-2, may represent the core histones or histone H1 degradation products. NANUC-1 and the negative control anti-tetanus toxoid antibody were not reactive with any protein species on Western blots.

In cell types other than neutrophils, histone H1 reactivity was detected in both whole cell lysate and nuclear fraction but not in the cytoplasmic fraction. In contrast, neutrophil nuclear fraction revealed a large amount of NANUC-2 reactive histone H1, but no reactivity was seen in whole cell lysate prepared from the same cells. The lack of reactivity in neutrophil whole cell lysate can be a result of very rapid degradation of histone H1 by proteases found in neutrophilic granules not in other cells such as Molt-4 cells (see Figure 4).

EXAMPLE II

IDENTIFICATION OF pANCA-REACTIVE H1 FRAGMENTS

This example demonstrates that particular fragments of histone H1 are reactive with the NANUC-2 antibody.

To further characterize the pANCA-reactive histone H1 epitope, purified protein was subjected to chemical cleavage with N-N bromosuccinamide (NBS) and proteolysis with chymotryspin, and reactivity of the resulting fragments was analyzed by silver staining and immunoblotting. NBS cleavage of H1 resulted in two fragments: an N-terminal 73 amino acid fragment (SEQ ID NO: 12) and a C-terminal 147 amino acid fragment (SEQ ID NO: 13). Because of the extremely charged nature of these fragments, the apparent mobilities of the 73 and 147 amino acid fragments are 23 and 11 kD, respectively. Immunoblot analysis revealed that only the larger 147 amino acid fragment (SEQ ID NO: 13) was reactive with NANUC-2, indicating that a pANCA-reactive epitope lies within the carboxy-terminal 147 amino acids. Chymotrypsin digests the N-terminal portion of the 147 amino carboxy-terminal fragment, producing a fragment with an apparent molecular weight of 17 kD. Immunoblots of chymotrypsin-digested H1 revealed NANUC-2 reactivity only with the carboxy-terminal fragment, thereby narrowing the pANCA-reactive epitope to the carboxy-terminal 113 amino acids (SEQ ID NO: 14). As a control to show that the nonreactive N-terminal fragments were properly transferred, the same blots showed reactivity with a rabbit anti-H1-3 polyclonal specific for the N-terminal fragment (see Parseghian et al., Chromosoma 103:198 (1994) and Parseghian et al., Chrom. Res. 1:127

(1993), each of which is incorporated herein by reference).

H1 epitopes were mapped using N-N bromosuccinamide and chymotrypsin proteolysis with a procedure modified from Parseghian et al., *supra*, 1993; Sherod et al., J. Biol Chem. 12:3923 (1974) and Costa et al., Clin. Exp. Immunol. 63: 608 (1986), each of which is incorporated herein by reference. Briefly, in a 10 µl reaction, 12 µg of bone marrow histone H1 was cleaved with 0.85 µg N-N bromosuccinamide (Sigma) in 0.9 N acetic acid. The reaction was terminated at varying time points by transferring 2.5 µL aliquots into 7.5 µl stop buffer (O.125 M Tris Cl pH 7.6 with 10.7 µg tyrosine (Sigma)). Chymotrypsin cleavage was performed in a 10 µl reaction volume by incubating 6 µg bone marrow histone H1 with 0.01 µg chymotrypsin (Boehringer Mannheim, Indianapolis, IN) in 0.1 M Tris-Cl pH 8.0, 10 mM CaCl. Reactions were stopped at various time points by addition of 1 µl 20 mM phenyl methyl sulfonyl fluoride (PMSF; Boehringer Mannheim), and 1.5 µg of NBS and chymotryptic H1 fragments diluted in Laemli buffer were run on 13% acrylamide gels using a Biorad mini gel apparatus, transferred to nitrocellulose, and immunoblotted as described above. Gels were silver stained using a Biorad silver stain kit (Biorad, Richmond, CA).

EXAMPLE III

IDENTIFICATION OF pANCA-REACTIVE PEPTIDES DERIVED FROM HISTONE H1

This example demonstrates that synthetic peptides spanning histone H1 can be assayed for NANUC-1 and NANUC-2 binding to identify pANCA-reactive peptides.

Overlapping 15-mer peptides that spanned the C-terminal 109 amino acids of the human $H1^{s}$-3 gene product with an N-terminal biotin were synthesized (P. Allen, Washington University, St. Louis). Each of the peptides overlapped adjacent peptide sequences by five amino acids except for peptide SEQ ID NO: 25. The eleven peptide sequences are shown in Table 2.

Peptides were tested for binding to 1.0, 3.0 and 10.0 µg/ml NANUC-1, NANUC-2 and negative control anti-tetanus toxoid antibody P313 (anti-TT). As shown in Figure 5, peptide SEQ ID NO: 20 was distinguished among the 11 peptides assayed by significant binding to NANUC-1 and NANUC-2 (OD_{405} of approximately 0. 5; significant at 1.0 µg/ml) compared to background levels observed with the rFab negative control anti-TT antibody, yielding an OD_{405} of less than 0.1. Neither of the two adjacent, overlapping peptides SEQ ID NO: 19 or SEQ ID NO: 21 showed significant binding to both NANUC antibodies. Peptide SEQ ID NO: 17 also reacted with NANUC-1 and NANUC-1; however, this binding was weaker (OD_{405} of approximately 0.25 for both NANUC antibodies) than the reactivity seen with peptide SEQ ID NO: 20.

Table 2	
Histone H1 peptide sequences	
SEQ ID NO:	Amino acid sequence
SEQ ID NO: 15	FKLNKKAASGEAKPK
SEQ ID NO: 16	EAKPKVKKAGGTKPK
SEQ ID NO: 17	GTKPKKPVGAAKKPK
SEQ ID NO: 18	AKKPKKAAGGATPKK
SEQ ID NO: 19	ATPKKSAKKTPKKAK
SEQ ID NO: 20	PKKAKKPAAATVTKK
SEQ ID NO: 21	TVTKKVAKSPKKAKV
SEQ ID NO: 22	KKAKVAKPKKAAKSA
SEQ ID NO: 23	AAKSAAKAVKPKAAK
SEQ ID NO: 24	PKAAKPKVVKPKKAA
SEQ ID NO: 25	KPKVVKPKKAAPKKK

These data indicate that histone H1 peptide PKKAKKPAAATVTKK (SEQ ID NO: 20) is specifically reactive with two distinct UCpANCA monoclonal antibodies. Because adjacent peptides lack activity, the pANCA reactivity of peptide SEQ ID NO: 20 may depend on its unique internal linear amino acid sequence KPAAA (SEQ ID NO: 26) or may depend on the unique conformation of the peptide sequence SEQ ID NO: 20 in its entirety.

The eleven H1 peptides SEQ ID NOS: 15 to 25 were assayed for reactivity with UC pANCA monoclonal antibodies NANUC-1 and NANUC-2 as follows. ELISA wells were coated with 50 µl solution of the H1 peptide of interest (at a concentration of 250 µg/ml) in carbonate buffer, pH 9.6, overnight at 4°C. Wells were blocked with phosphate buffered saline/0.5% Tween-20/500 µg/ml bovine serum albumin (BSA; SIGMA) for 1 hour at

room temperature. Wells were washed five times with 0.05% Tween-20 in PBS (wash buffer), then reacted with rFab antibody diluted in wash buffer at indicated concentrations for 2 hours at room temperature. Plates were washed five times, and immunocomplexes detected subsequently with 0.05% alkaline phosphatase-conjugated goat anti-human Fab (Pierce) in wash buffer for 1 hour at room temperature. After washing, the samples were reacted with BCIP-NBT substrate (SIGMA). Figure 5 shows the absorbance at 405 nm (OD_{405}) after normalization for background binding due to reactivity with secondary antibody alone.

EXAMPLE IV

IDENTIFICATION OF pANCA-REACTIVE PEPTIDES DERIVED FROM HISTONE H1

This example demonstrates that fragments of histone H1 isoform H1.5 bind NANUC-2, thus identifying these fragments as pANCA-reactive fragments of histone H1.

Five recombinant fragments of human histone H1 isoform H1.5 (SEQ ID NO: 32) that spanned the C-terminal 158 amino acids of the H1.5 protein (amino acids 69 to 226 of SEQ ID NO: 32) with an N-terminal biotin were synthesized. The five fragments were as follows: SEQ ID NO: 37 (amino acids 172 to 184 of H1.5); SEQ ID NO: 38 (amino acids 69 to 184 of H1.5); SEQ ID NO: 39 (amino acids 69 to 171 of H1.5; SEQ ID NO: 40 (amino acids 69 to 226 of H1.5); and SEQ ID NO: 41 (amino acids 172 to 226 of H1.5).

Each of these five fragments bound selectively to NANUC-2 but did not bind to a control anti-tetanus

toxoid antibody. The binding of SEQ ID NOS: 37, 38, 40 and 41 to NANUC-2 indicates the presence of a pANCA-reactive epitope of histone H1 that is in the region of amino acids 172 to 184 of histone H1.5. This region of histone isoform H1.5 is highly conserved among varying species and isoforms of histone H1 and is present, for example, in histone $H1^s$-3. The binding of SEQ ID NO: 39 to NANUC-2 indicates the presence of an additional pANCA-reactive epitope of histone H1 isoform H1.5 outside amino acids 172 to 184, within amino acids 69 to 171 of H1.5. In sum, these results demonstrate that histone H1 contains at least two distinct pANCA-reactive epitopes.

EXAMPLE V

IDENTIFICATION OF ULCERATIVE COLITIS pANCA HISTONE H1-LIKE TARGET ANTIGENS

This example demonstrates that histone H1-like antigens present in a variety of Mycobacterial species are reactive with the NANUC-2 antibody.

A. A mycobacterial histone H1-like antigen is a pANCA reactive protein

BLAST analysis was used to search the microbial databases for linear peptide homologues of the C-terminal random coil domain of human histone H1 isoform H1.5 (SEQ ID NO: 32). This analysis revealed homologous sequences reported in the *M. tuberculosis* genome database as anonymous open reading frames (ORFs).

In view of these homologous *M. tuberculosis* ORFs, seven Mycobacterial strains from five Mycobacterial species were obtained from the American Type Culture

Collection and from St. Mary's Hospital Medical School (London, UK). The Mycobacteria were grown using the media and growth conditions shown in Figure 6. Whole cell extracts were prepared and analyzed by Western analysis using the NANUC-2 antibody essentially as described in Example I. As shown in Figure 7, the NANUC-2 antibody detected a single 30-32 kDa protein, with a slightly varying size in each of the Mycobacterial species.

The pANCA-reactive 30-32 kDa protein from *M. avium paratuberculosis* was isolated by SDS-PAGE electrophoresis and PVDF membrane transfer. As shown in Figure 8, the sequence of the first 18 amino acids of the N-terminal fragment (SEQ ID NO: 31) obtained by peptide sequencing was nearly identical to the amino-terminal sequence of a predicted protein of 214 amino acids from the *M. tuberculosis* genome (SEQ ID NO: 27). The two amino acid differences in the N-terminal 18 amino acid sequence can reflect sequencing artifacts or sequence polymorphisms between *M. avium paratuberculosis* and *M. tuberculosis*. Alignment of the entire 214 amino acid *M. tuberculosis* protein (SEQ ID NO: 27) with human histone H1 isoform H1.5 (SEQ ID NO: 32) revealed 48% sequence similarity.

B. Recombinant HupB is recognized by NANUC-2

The 214 amino acid *M. tuberculosis* protein (HupB; SEQ ID NO: 27) was subcloned into pGEX-KG as a GST fusion under control of a β-gal promoter and expressed in *E. coli* as follows. *M. tuberculosis* Erdman strain was cultured, and genomic DNA to be used as a template was extracted by phenol-chloroform. Two sets of nested oligonucleotide primers were designed to amplify the

protein based on the DNA sequence available as Accession number Z83018 with additional EcoRI and HindIII sites. The PCR product was ligated as a carboxy-terminal fusion to the GST gene of pGEX-KG. The GST fusion plasmids were transformed into XL-1 blue *E. coli* (Stratagene, La Jolla, CA); positive clones were validated by PCR amplification and sequencing using pGEX sequencing primers (Pharmacia).

Recombinant GST fusion proteins were produced as follows. Vectors were freshly transformed into *E. coli* XL-1 blue, XL-21 and XL-21 blue. For expression, 10 ml of a 24 hour bacterial culture was inoculated into 0.5 Luria-Bertani broth with ampicillin (0.1 mg/ml), cultured at 37 °C with shaking at 200 rpm to mid-log phase (0.6 OD_{600}). Cultures were harvested by centrifugation and resuspended in lysis buffer (50 mM Tris-CL pH 7.5, 300 mM NaCl, 10 mM EDTA, 0.1% SDS and protease inhibitors). Cells were lysed by two periods of one minute sonication at 50% intensity using a Misonix Ultrasonic Processor (Misonix, Farmingdale, NY). The soluble fraction of each lysate was isolated by centrifugation (12,000 x g for 18 minutes). Purified recombinant proteins were quantified by Bradford analysis, and analyzed by ELISA or gel electrophoresis followed by silver staining or immunoblotting.

Production of the protein proved difficult since the protein showed a high level of toxicity resulting in total cell death or a slow growth rate. XL-1 Blue cells were least affected by the gene product, growing at about 1.2 hour Td. In addition, efficient protein purification was hindered by the limited solubility and high susceptibility of the recombinant protein to proteolysis. However, high levels of expression allowed recombinant protein purification up to

50% of total protein by SDS-PAGE and protein staining. The recombinant fusion protein containing the 214 amino acid *M. tuberculosis* antigen (HupB; SEQ ID NO: 27) migrated at about 60 kDa apparent molecular weight as expected; its identity was confirmed by probing with a mouse anti-GST monoclonal.

Western analysis indicated strong reactivity with NANUC-2. Reactivity was specific, since no NANUC-2 reactivity was detected to the wild type GST protein, and no reactivity was seen with the negative control Fab 313 antibody. These results confirm that a histone H1-like antigen can be the 214 amino acid *Mycobacterial* antigen SEQ ID NO: 27, recently designated HupB. Furthermore, analysis of HupB proteolytic laddering indicated significant loss in reactivity for species of 125 amino acids of less, localizing the NANUC-2 reactive portion of HupB to the carboxy-terminal 90 amino acids (residues 125 to 214 of SEQ ID NO: 27).

C. A Mycobacterial histone H1-like antigen is differentially reactive with normal and pANCA-positive UC patient sera

Samples of human sera are taken from patients diagnosed with UC and from control normal individuals. Each UC sample is assayed against human PMN (neutrophil) for the presence of UC pANCA. pANCA-positive UC sera and normal sera are then assayed for reactivity against the *M. tuberculosis* 214 amino acid histone H1-like antigen (SEQ ID NO: 27). The results show that a significantly greater percentage of UC pANCA-positive samples are reactive with the histone H1-like antigen as compared to the percentage of normal samples that react. These results demonstrate that a histone H1-like microbial

protein can be a UC pANCA antigen useful in the diagnosis of ulcerative colitis.

EXAMPLE VI

IDENTIFICATION OF ULCERATIVE COLITIS pANCA PORIN AND *BACTEROIDES* TARGET ANTIGENS

This example demonstrates that porin and *Bacteroides* antigens expressed by enteric bacteria from UC patients are reactive with the NANUC-2 antibody.

A. Enteric *E. coli* from UC patients express a pANCA-reactive antigen

The disease-specific immune response exemplified by UC pANCA can be elicited by cross-reactive proteins of particular enteric bacteria. To isolate these bacteria, colonic biopsy specimens were obtained from three patients diagnosed with UC. The specimens were minced, and the colonic bacteria cultured using varying oxygen availability and culture media. After harvesting bacteria from the cultures, bacterial extracts were tested by Western analysis with the NANUC-2 antibody essentially as described above.

Cross-reactive proteins were only identified in the anaerobic cultures. The NANUC-2-reactive cultures were plated, and single colonies isolated and individually screened for UC pANCA antigen expression by Western analysis. Seven positive clones were isolated and identified by 16S rRNA sequencing essentially as described in Wilson and Blitchington, Applied and Environ. Microbiol. 62:2273-2278 (1996).

In particular, five clones were identified by 16S rRNA-typing as members of the *E. coli* species (P1Bc5, P1Bc9, P2c2, P2c5 and P2Lc2). As shown in Figure 9, each of the five types of *E. coli* expressed a pANCA-reactive protein of about 35 kDa. These pANCA-reactive proteins could be divided into larger and smaller types, which were isolated by SDS-PAGE electrophoresis and PVDF membrane transfer. N-terminal amino acid sequencing of the larger and smaller pANCA-reactive proteins revealed an identical 19-mer peptide (SEQ ID NO: 33).

BLAST analysis revealed that this 19 amino acid sequence (SEQ ID NO: 33) is shared by a set of three closely related putative ORFs of the porin outer-membrane protein family (SEQ ID NOS: 28, 29 and 30; see Figure 10). Shown in Figure 12 is the CLUSTAL and BEST-FIT alignment of one such porin antigen (SEQ ID NO: 28) with histone H1.5 (SEQ ID NO: 32). This alignment reveals that porin antigens lack detectable linear sequence homology with histone H1.5 (SEQ ID NO: 32).

B. A porin antigen reactive with NANUC-1 or NANUC-2 can be a ompC porin

Three *E. coli* mutants, ompC-, ompF- and ompC-/ ompF- were obtained from Rajeev Misra at the University of Arizona. Extracts were prepared by boiling 20 µl of 0.6 OD_{600} bacterial suspension in the same volume Laemmli sample buffer; 20 µg were electrophoresed on SDS-PAGE. Western analysis was performed with NANUC-2 as described above.

The results of western analysis indicated that the 35 kDa porin antigen was absent from the OmpC- OmpF-

double mutant as well as from the OmpC- single mutant. However, the NANUC-reactive porin antigen was present in the OmpF- strain as well as in control wild type *E. coli* cultures. These results demonstrate that an *E. coli* porin antigen of the invention can be OmpC or a OmpC homolog.

C. A porin antigen expressed in *E. coli* is differentially reactive with normal and UC pANCA-positive patient sera

Samples of human sera are taken from patients diagnosed with UC and from control normal individuals. Each UC sample is assayed against human PMN (neutrophil) for the presence of UC pANCA. pANCA-positive UC sera and normal sera are then assayed for reactivity against the *E. coli* porin antigens described above (SEQ ID NOS: 28, 29 and 30). The results show that a significantly greater percentage of UC pANCA-positive samples are reactive with the porin antigen as compared to the percentage of normal samples that react. These results demonstrate that a porin antigen, such as one of the *E. coli* porin antigens described above (SEQ ID NOS: 28, 29 and 30), can be a UC pANCA antigen useful in the diagnosis of ulcerative colitis.

D. Enteric Bacteroides from UC patients express a UC pANCA-reactive antigen

Two clones were identified by 16S rRNA-typing as described above as members of the *Bacteroides caccae* family (P2Lc3 and P2Lc5). As shown in Figure 9B, these family members expressed a pANCA-reactive protein of about 100 kDa. Western analysis showed that the pANCA-reactive 100 kDa Bacteroides antigen was

selectively expressed in Bacteroides but not expressed, for example, in *E. coli*.

Different *Bacteroides* species isolated from CD patients were obtained from Clin-Micro UCLA. After growing the clinical isolates on blood agar plates and washing with 5 ml 0.15 NaCl, the cells were centrifuged at 200 rpm from 10 minutes and resuspended in 50 mM TrisCl pH 7.2 to bring the OD_{600} to 2. For western analysis, 5 µl of each sample was electrophoresed on 12% SDS-PAGE. Gels were transferred and probed with NANUC-2 as described above.

A pANCA-reactive 100 kDa protein was present in *B. caccae* extracts (strain 43185 and strain P2LC3). In addition, a faster migrating pANCA-reactive antigen of about 75 kDa was present in several *B. thetaiotaomicron* strains (4552, 4562 and 4578) although not in *B. thetaiotaomicron* strain 4536. No pANCA-reactive antigens were observed by western analysis in *B. vulgatus* strains 4579, LGI, LGI-33, or CDT-6; *B. fragilis* strains 4570 and 4556; or in *B. ureolyticus* strain 3955-3. These results indicate that a Bacteroides antigen of the invention can be differentially expressed in particular *Bacteroides* strains such as *B. caccae* or *B. thetaiotaomicron*.

E. Sequence from the Bacteroides 100 kDa antigen

The P2LC3 *B. caccae* isolate was used as a source for large scale protein purification. Large scale plate cultures (180 plates) or fermentation cultures (40 L) were purified using 50% ammonium sulfate precipitation and DE52 ion exhange column chromatography at pH 8 and eluted using a NaCl gradient (Harth et al., Infect.

Immunity 65:2321-2328 (1997)). The positive fractions were identified by western analysis, at 0.2-0.4 M NaCl elution.

The ~100 kDa Bacteroides antigen was detected as a set of five distinct bands of ~97 kDa, 101 kDa, 102 kDa, 105 kDa and 95 kDa. Following Coomassie blue staining, the four lower molecular weight bands were analyzed at The Keck Institute, Yale. The proteins were digested in gel with trypsin per standard Keck Institute protocols. Fragments were extracted from the gel, partially dried, and subject to MALDI-MS analysis. The MALDI-MS profiles of the tryptic digests were compared to available profiles of known proteins. No matches for the samples were identified that satisfied the Keck Institute criteria for identification of an unknown protein.

Tryptic digests were subsequently separated by size exclusion HPLC on a Sephadex 200 HR 10/30 column with separation ranging from 10 kDa to 200 kDa (Pharmacia). Three peaks (71, 35 and 45) were N-terminally sequenced by Edman degradation, and the sequences shown in Table 3 obtained.

Analysis of the peptide sequences obtained for peaks 71, 35 and 45 indicated that the three peptides did not overlap (see Table 3). Blast searching with each of the peptide sequences yielded only minimal matches (fewer than 7 amino acids with p_{value} approaching 1). None of the proteins identified by minimal matches showed homology to the sequence obtained from more than one peak. These results confirm the analysis performed with the MALDI-MS tryptic digest profiles, indicating that the 100 kDa pANCA-reactive Bacteroides antigen is a novel protein.

Table 3

Sequence data for Bacteroides 100 kDa antigen

Sample	Peak	Purity	Size	SIN	Amino Acid Seq.
97 kDa	71	100%	1162.9	44	DPSLAIFGVR
"	"	"	"	45	DPSSLAIFGVR
102 kDa	35	100%	1199.1	46	GPSEADAFYNC
"	"	"	"	47	GPSYADAFYNC
101 kDa	45	87%	1007.7	48	YFLASYR
"	"	"	"	49	YFLSASYR
"	"	"	"	50	YFLAGNGE
"	"	"	"	51	YFLSAGNGE
"	"	"	"	52	AFLASYR
"	"	"	"	53	AFLSASYR
"	"	"	"	54	AFLAGNGE
"	"	"	"	55	AFLSAGNGE
"	"	"	"	56	SFLASYR
"	"	"	"	57	SFLSASYR
"	"	"	"	58	SFLAGNGE
"	"	"	"	59	SFLSAGNGE

F. An antigen expressed in Bacteroides is differentially reactive with normal and UC pANCA-positive patient

Samples of human sera are taken from patients diagnosed with UC and from control normal patients. Each UC sample is assayed against human PMN (neutrophil) for the presence of UC pANCA. pANCA-positive UC sera and normal sera are then assayed for reactivity against a protein of about 100 kDa that is isolated from *Bacteroides caccae* using SDS-PAGE electrophoresis and

PVDF membrane transfer. The results show that a significantly greater percentage of UC pANCA-positive samples are reactive with the Bacteroides antigen as compared to the percentage of normal samples that react. These results demonstrate that a Bacteroides antigen, such as the 100 kDa antigen isolated from *Bacteroides caccae*, can be a UC pANCA antigen useful in the diagnosis of ulcerative colitis.

All journal article, reference and patent citations provided above, in parentheses or otherwise, whether previously stated or not, are incorporated herein by reference in their entirety.

Although the invention has been described with reference to the examples above, it should be understood that various modifications can be made without departing from the spirit of the invention. Accordingly, the invention is limited only by the following claims.

We claim:

1. A method of diagnosing ulcerative colitis (UC) in a subject suspected of having inflammatory bowel disease, comprising:

(a) obtaining a sample from said subject;

(b) contacting said sample with a histone H1-like antigen, or pANCA-reactive fragment thereof, under conditions suitable to form a complex of said histone H1-like antigen, or said pANCA-reactive fragment thereof, and antibody to said histone H1-like antigen; and

(c) detecting the presence or absence of said complex,

wherein the presence of said complex indicates that said subject has UC.

2. The method of claim 1, wherein said histone H1-like antigen comprises a protein immunoreactive with NANUC-2 and having an amino acid sequence having at least 65% amino acid identity with SEQ ID NO: 27.

3. A method of inducing tolerance in a pANCA-positive patient with UC, comprising administering an effective dose of histone H1-like antigen, or tolerogenic fragment thereof, to said pANCA-positive patient with UC.

4. The method of claim 3, wherein said histone H1-like antigen comprises a protein immunoreactive with NANUC-2 and having an amino acid sequence having at least 65% amino acid identity with SEQ ID NO: 27.

5. A composition comprising histone H1-like antigen, or tolerogenic fragment thereof, combined with a tolerogizing molecule.

6. The composition of claim 5, wherein said histone H1-like antigen comprises a protein immunoreactive with NANUC-2 and having an amino acid sequence having at least 65% amino acid identity with SEQ ID NO: 27.

7. A method of diagnosing UC in a subject suspected of having inflammatory bowel disease, comprising:

(a) obtaining a sample from said subject;

(b) contacting said sample with a porin antigen, or pANCA-reactive fragment thereof, under conditions suitable to form a complex of said porin antigen, or said pANCA-reactive fragment thereof, and antibody to said porin antigen; and

(c) detecting the presence or absence of said complex,

wherein the presence of said complex indicates that said subject has UC.

8. The method of claim 7, wherein said porin antigen comprises a protein immunoreactive with NANUC-2 and having an amino acid sequence having at least 65% amino acid identity with a sequence selected from the group consisting of SEQ ID NO: 28, SEQ ID NO: 29 and SEQ ID NO: 30.

9. A method of inducing tolerance in a pANCA-positive patient with UC, comprising administering an effective dose of a porin antigen, or tolerogenic fragment thereof, to said pANCA-positive patient with UC.

10. The method of claim 9, wherein said porin antigen comprises a protein immunoreactive with NANUC-2 and having an amino acid sequence having at least 65% amino acid identity with a sequence selected from the group consisting of SEQ ID NO: 28, SEQ ID NO: 29 and SEQ ID NO: 30.

11. A composition comprising a porin antigen, or tolerogenic fragment thereof, combined with a tolerogizing molecule.

12. The composition of claim 11, wherein said porin antigen comprises a protein immunoreactive with NANUC-2 and having an amino acid sequence having at least 65% amino acid sequence identity with a sequence selected from the group consisting of SEQ ID NO: 28, SEQ ID NO: 29 and SEQ ID NO: 30.

13. A method of diagnosing UC in a subject suspected of having inflammatory bowel disease, comprising:

(a) obtaining a sample from said subject;

(b) contacting said sample with a Bacteroides antigen, or pANCA-reactive fragment thereof, under conditions suitable to form a complex of said Bacteroides antigen, or said pANCA-reactive fragment thereof, and antibody to said Bacteroides antigen; and

(c) detecting the presence or absence of said complex,

wherein the presence of said complex indicates that said subject has UC.

14. The method of claim 13, wherein said Bacteroides antigen comprises a *Bacteroides caccae* protein immunoreactive with NANUC-2 and having a molecular weight of about 100 kDa by SDS-PAGE electrophoresis.

15. A method of inducing tolerance in a pANCA-positive patient with UC, comprising administering an effective dose of a Bacteroides antigen, or tolerogenic fragment thereof, to said pANCA-positive patient with UC.

16. The method of claim 15, wherein said Bacteroides antigen comprises a *Bacteroides caccae* protein immunoreactive with NANUC-2 and having a molecular weight of about 100 kDa by SDS-PAGE electrophoresis.

17. A composition comprising a Bacteroides antigen, or tolerogenic fragment thereof, combined with a tolerogizing molecule.

18. The method of claim 17, wherein said Bacteroides antigen comprises a *Bacteroides caccae* protein immunoreactive with NANUC-2 and having a molecular weight of about 100 kDa by SDS-PAGE electrophoresis.

19. A method of identifying an agent useful for treating UC, comprising:

(a) obtaining a sample of enteric bacteria from a patient with UC;

(b) isolating from said sample a bacterial species that expresses a pANCA-reactive antigen;

(c) contacting said bacterial species with an agent; and

(d) assaying for reduced growth or viability of said bacterial species,

wherein said reduced growth or viability of said bacterial species indicates that said agent is an agent useful in treating UC.

20. The method of claim 19, wherein said bacterial species that expresses a pANCA-reactive antigen is a member of a genus selected from the group of genera consisting of Mycobacteria, Escherichia and Bacteroides.

21. The method of claim 20, wherein said bacterial species that expresses a pANCA-reactive antigen is a Mycobacterial species that is selected from the group consisting of *Mycobacterium tuberculosis, Mycobacterium bovis, Mycobacterium smegmatis 1-2c, Mycobacterium avium* and *Mycobacterium avium paratuberculosis.*

22. The method of claim 20, wherein said bacterial species that expresses a pANCA-reactive antigen is *Escherichia coli.*

23. The method of claim 20, wherein said bacterial species that expresses a pANCA-reactive antigen is *Bacteroides caccae.*

SEQUENCE LISTING

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<120> Diagnosis, Prevention and Treatment of Ulcerative Colitis, and Clinical Subtypes Thereof, Using Microbial UC pANCA Antigens

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35 40 45

Ala Ser Lys Glu Arg Ser Gly Val Ser Leu Ala Ala Leu Lys Lys Ala
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Lys Leu Gly Leu Lys Ser Leu Val Ser Lys Gly Thr Leu Val Gln Thr
                 85                  90                  95

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            100                 105                 110

Ser Gly Glu Gly Lys Pro Lys Ala Lys Lys Ala Gly Ala Ala Lys Pro
        115                 120                 125

Arg Lys Pro Ala Gly Ala Ala Lys Lys Pro Lys Lys Val Ala Gly Ala
    130                 135                 140

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145                 150                 155                 160

Pro Ala Thr Ala Ala Gly Thr Lys Lys Val Ala Lys Ser Ala Lys Lys
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Val Lys Thr Pro Gln Pro Lys Lys Ala Ala Lys Ser Pro Ala Lys Ala
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Val Thr Lys Ala Lys Lys Ala Ala Pro Lys Lys Lys
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Lys Lys Ala Leu Ala Ala Gly Gly Tyr Asp Val Glu Lys Asn Asn Ser
65                  70                  75                  80

Arg Ile Lys Leu Gly Leu Lys Ser Leu Val Ser Lys Gly Thr Leu Val
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Gln Thr Lys Gly Thr Gly Ala Ser Gly Ser Phe Lys Leu Asn Lys Lys
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Ala Ala Ser Gly Glu Ala Lys Pro Lys Ala Lys Lys Ala Gly Ala Ala
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Lys Ala Lys Lys Pro Ala Gly Ala Thr Pro Lys Lys Ala Lys Lys Ala
    130                 135                 140

Ala Gly Ala Lys Lys Ala Val Lys Lys Thr Pro Lys Lys Ala Lys Lys
145                 150                 155                 160

Pro Ala Ala Ala Gly Val Lys Lys Val Ala Lys Ser Pro Lys Lys Ala
                165                 170                 175

Lys Ala Ala Ala Lys Pro Lys Lys Ala Thr Lys Ser Pro Ala Lys Pro
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         35                  40                  45

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     50                  55                  60

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 65                  70                  75                  80

Leu Gly Leu Lys Ser Leu Val Ser Lys Gly Thr Leu Val Gln Thr Lys
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Gly Thr Gly Ala Ser Gly Ser Phe Lys Leu Asn Lys Lys Ala Ala Ser
            100                 105                 110

Gly Glu Ala Lys Pro Lys Ala Lys Lys Ala Gly Ala Ala Lys Ala Lys
        115                 120                 125

Lys Pro Ala Gly Ala Ala Lys Lys Pro Lys Lys Ala Thr Gly Ala Ala
    130                 135                 140

Thr Pro Lys Lys Ser Ala Lys Lys Thr Pro Lys Lys Ala Lys Lys Pro
145                 150                 155                 160

Ala Ala Ala Ala Gly Ala Lys Lys Ala Lys Ser Pro Lys Lys Ala Lys
                165                 170                 175

Ala Ala Lys Pro Lys Lys Ala Pro Lys Ser Pro Ala Lys Ala Lys Ala
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         35                  40                  45

Ile Gln Lys Tyr Ile Lys Ser His Tyr Lys Val Gly Glu Asn Ala Asp
     50                  55                  60

Ser Gln Ile Lys Leu Ser Ile Lys Arg Leu Val Thr Thr Gly Val Leu
 65                  70                  75                  80

Lys Gln Thr Lys Gly Val Gly Ala Ser Gly Ser Phe Arg Leu Ala Lys
                 85                  90                  95

Ser Asp Glu Pro Lys Lys Ser Val Ala Phe Lys Lys Thr Lys Lys Glu
            100                 105                 110

Ile Lys Lys Val Ala Thr Pro Lys Lys Ala Ser Lys Pro Lys Lys Ala
        115                 120                 125

Ala Ser Lys Ala Pro Thr Lys Lys Pro Lys Ala Thr Pro Val Lys Lys
    130                 135                 140

Ala Lys Lys Lys Leu Ala Ala Thr Pro Lys Lys Ala Lys Lys Pro Lys
145                 150                 155                 160

Thr Val Lys Ala Lys Pro Val Lys Ala Ser Lys Pro Lys Lys Ala Lys
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Pro Val Lys Pro Lys Ala Lys Ser Ser Ala Lys Arg Ala Gly Lys Lys
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Lys
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         35                  40                  45

Glu Ala Leu Ser Val Ser Gln Glu Arg Val Gly Met Ser Leu Val Ala
     50                  55                  60

Leu Lys Lys Ala Leu Ala Ala Ala Gly Tyr Asp Val Glu Lys Asn Asn
 65                  70                  75                  80

Ser Arg Ile Lys Leu Ser Leu Lys Ser Leu Val Asn Lys Gly Ile Leu
                 85                  90                  95

Val Gln Thr Arg Gly Thr Gly Ala Ser Gly Ser Phe Lys Leu Ser Lys
            100                 105                 110

Lys Val Ile Pro Lys Ser Thr Arg Ser Lys Ala Lys Lys Ser Val Ser
        115                 120                 125

Ala Lys Thr Lys Lys Leu Val Leu Ser Arg Asp Ser Lys Ser Pro Lys
    130                 135                 140

Thr Ala Lys Thr Asn Lys Arg Ala Lys Lys Pro Arg Ala Thr Thr Pro
145                 150                 155                 160

Lys Thr Val Arg Ser Gly Arg Lys Ala Lys Gly Ala Lys Gly Lys Gln
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Leu Gln Ser Ser Gly Leu Tyr Ser Leu Ser Ser Val Val Thr Val Pro
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Ser Ser Ser Leu Gly Thr Gln Thr Tyr Ile Cys Asn Val Asn His Lys
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    130                 135                 140

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Lys Val Gln Trp Lys Val Asp Asn Ala Leu Gln Ser Gly Asn Ser Gln
145                 150                 155                 160

gag agt gtc aca gag cag gac agc aag gac agc acc tac agc ctc agc   528
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Thr Ala Ser Val Val Cys Leu Leu Asn Asn Phe Tyr Pro Arg Glu Ala
130 135 140

Lys Val Gln Trp Lys Val Asp Asn Ala Leu Gln Ser Gly Asn Ser Gln
145 150 155 160

Glu Ser Val Thr Glu Gln Asp Ser Lys Asp Ser Thr Tyr Ser Leu Ser
165 170 175

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35 40 45

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50 55 60

Pro Lys Lys Val Ala Gly Ala Ala Thr Pro Lys Lys Ser Ile Lys Lys
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Met Asn Lys Ala Glu Leu Ile Asp Val Leu Thr Gln Lys Leu Gly Ser
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Asp Arg Arg Gln Ala Thr Ala Ala Val Glu Asn Val Val Asp Thr Ile
            20                  25                  30

Val Arg Ala Val His Lys Gly Asp Ser Val Thr Ile Thr Gly Phe Gly
        35                  40                  45

Val Phe Glu Gln Arg Arg Arg Ala Ala Arg Val Ala Arg Asn Pro Arg
    50                  55                  60

Thr Gly Glu Thr Val Lys Val Lys Pro Thr Ser Val Pro Ala Phe Arg
65                  70                  75                  80

Pro Gly Ala Gln Phe Lys Ala Val Val Ser Gly Ala Gln Arg Leu Pro
                85                  90                  95

Ala Glu Gly Pro Ala Val Lys Arg Gly Val Gly Ala Ser Ala Ala Lys
            100                 105                 110

Lys Val Ala Lys Lys Ala Pro Ala Lys Lys Ala Thr Lys Ala Ala Lys
        115                 120                 125

Lys Ala Ala Thr Lys Ala Pro Ala Arg Lys Ala Ala Thr Lys Ala Pro
    130                 135                 140

Ala Lys Lys Ala Ala Thr Lys Ala Pro Ala Lys Lys Ala Val Lys Ala
145                 150                 155                 160

Thr Lys Ser Pro Ala Lys Lys Val Thr Lys Ala Val Lys Lys Thr Ala
                165                 170                 175

Val Lys Ala Ser Val Arg Lys Ala Ala Thr Lys Ala Pro Ala Lys Lys
            180                 185                 190

Ala Ala Ala Lys Arg Pro Ala Thr Lys Ala Pro Ala Lys Lys Ala Thr
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Ala Arg Arg Gly Arg Lys
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 1               5                  10                  15

Gly Ala Ala His Ala Ala Glu Val Tyr Asn Lys Asp Gly Asn Lys Leu
            20                  25                  30

Asp Leu Tyr Gly Lys Val Asp Gly Leu His Tyr Phe Ser Asp Asn Ser
        35                  40                  45

Ala Lys Asp Gly Asp Gln Ser Tyr Ala Arg Leu Gly Phe Lys Gly Glu
    50                  55                  60

Thr Gln Ile Asn Asp Gln Leu Thr Gly Tyr Gly Gln Trp Glu Tyr Asn
65                  70                  75                  80

Ile Gln Ala Asn Asn Thr Glu Ser Ser Lys Asn Gln Ser Trp Thr Arg
                85                  90                  95

Leu Ala Phe Ala Gly Leu Lys Phe Ala Asp Tyr Gly Ser Phe Asp Tyr
            100                 105                 110

Gly Arg Asn Tyr Gly Val Met Tyr Asp Ile Glu Gly Trp Thr Asp Met
        115                 120                 125

Leu Pro Glu Phe Gly Gly Asp Ser Tyr Thr Asn Ala Asp Asn Phe Met
    130                 135                 140

Thr Gly Arg Ala Asn Gly Val Ala Thr Tyr Arg Asn Thr Asp Phe Phe
145                 150                 155                 160

Gly Leu Val Asn Gly Leu Asn Phe Ala Val Gln Tyr Gln Gly Asn Asn
                165                 170                 175

Glu Gly Ala Ser Asn Gly Gln Glu Gly Thr Asn Asn Gly Arg Asp Val
            180                 185                 190

Arg His Glu Asn Gly Asp Gly Trp Gly Leu Ser Thr Thr Tyr Asp Leu
        195                 200                 205

Gly Met Gly Phe Ser Ala Gly Ala Ala Tyr Thr Ser Ser Asp Arg Thr
    210                 215                 220
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Asn Asp Gln Val Asn His Thr Ala Ala Gly Gly Asp Lys Ala Asp Ala
225                 230                 235                 240

Trp Thr Ala Gly Leu Lys Tyr Asp Ala Asn Asn Ile Tyr Leu Ala Thr
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Met Tyr Ser Glu Thr Arg Asn Met Thr Pro Phe Gly Asp Ser Asp Tyr
            260                 265                 270

Ala Val Ala Asn Lys Thr Gln Asn Phe Glu Val Thr Ala Gly Tyr Gln
        275                 280                 285

Phe Asp Phe Gly Leu Arg Pro Ala Val Ser Phe Leu Met Ser Lys Gly
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Arg Asp Leu His Ala Ala Gly Gly Ala Asp Asn Pro Ala Gly Val Asp
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Asp Lys Asp
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Gly Ala Ala His Ala Ala Glu Val Tyr Asn Lys Asp Gly Asn Lys Leu
            20                  25                  30

Asp Leu Tyr Gly Lys Val Asp Gly Leu His Tyr Phe Ser Asp Asn Ser
        35                  40                  45

Ala Lys Asp Gly Asp Gln Ser Tyr Ala Arg Leu Gly Phe Lys Gly Glu
    50                  55                  60

Thr Gln Ile Asn Asp Gln Leu Thr Gly Tyr Gln Gln Trp Glu Tyr Asn
65                  70                  75                  80

Ile Gln Ala Asn Asn Thr Glu Ser Ser Lys Asn Gln Ser Trp Thr Arg
                85                  90                  95

Leu Ala Phe Ala Gly Leu Lys Phe Ala Asp Tyr Gly Ser Phe Asp Tyr
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100 105 110

Gly Arg Asn Tyr Gly Val Met Tyr Asp Ile Glu Gly Trp Thr Asp Met
115 120 125

Leu Pro Glu Phe Gly Gly Asp Ser Tyr Thr Asn Ala Asp Asn Phe Met
130 135 140

Thr Gly Arg Ala Asn Gly Val Ala Thr Tyr Arg Asn Thr Asp Phe Phe
145 150 155 160

Gly Leu Val Asn Gly Leu Asn Phe Ala Val Gln Tyr Gln Gly Asn Asn
165 170 175

Glu Gly Ala Ser Asn Gly Gln Glu Gly Thr Asn Asn Gly Arg Asp Val
180 185 190

Arg His Glu Asn Gly Asp Gly Trp Gly Leu Ser Thr Thr Tyr Asp Leu
195 200 205

Gly Met Gly Phe Ser Ala Gly Ala Ala Tyr Thr Ser Ser Asp Arg Thr
210 215 220

Asn Asp Gln Val Asn His Thr Ala Ala Gly Gly Asp Lys Ala Asp Ala
225 230 235 240

Trp Thr Ala Gly Leu Lys Tyr Asp Ala Asn Asn Ile Tyr Leu Ala Thr
245 250 255

Met Tyr Ser Glu Thr Arg Asn Met Thr Pro Phe Gly Asp Ser Asp Tyr
260 265 270

Ala Val Ala Asn Lys Thr Gln Asn Phe Glu Val Thr Ala Gln Tyr Gln
275 280 285

Phe Asp Phe Gly Leu Arg Pro Ala Val Ser Phe Leu Met Ser Lys Gly
290 295 300

Arg Asp Leu His Ala Ala Gly Gly Ala Asp Asn Pro Ala Gly Val Asp
305 310 315 320

Asp Lys Asp Leu Val Lys Tyr Ala Asp Ile Gly Ala Thr Tyr Tyr Phe
325 330 335

Asn Lys Asn Met Ser Thr Tyr Val Asp Tyr Lys Ile Asn Leu Ile Asp
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Glu Asp Asp Ser Phe Tyr Ala Ala Asn Gly Ile Ser Thr Asp Asp Ile

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Val Ala Leu Gly Leu Val Tyr Gln Phe
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35 40 45

Asp Val Asp Gly Asp Gln Thr Tyr Met Arg Leu Gly Phe Lys Gly Glu
50 55 60

Thr Gln Val Thr Asp Gln Leu Thr Gly Tyr Gly Gln Trp Glu Tyr Gln
65 70 75 80

Ile Gln Gly Asn Ser Ala Glu Asn Glu Asn Asn Ser Trp Thr Arg Val
85 90 95

Ala Phe Ala Gly Leu Lys Phe Gln Asp Val Gly Ser Phe Asp Tyr Gly
100 105 110

Arg Asn Tyr Gly Val Val Tyr Asp Val Thr Ser Trp Thr Asp Val Leu
115 120 125

Pro Glu Phe Gly Gly Asp Thr Tyr Gly Ser Asp Asn Phe Met Gln Gln
130 135 140

Arg Gly Asn Gly Phe Ala Thr Tyr Arg Asn Thr Asp Phe Phe Gly Leu
145 150 155 160

Val Asp Gly Leu Asn Phe Ala Val Gln Tyr Gln Gly Lys Asn Gly Asn
165 170 175

Pro Ser Gly Glu Gly Phe Thr Ser Gly Val Thr Asn Asn Gly Arg Asp
180 185 190

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Ala Leu Arg Gln Asn Gly Asp Gly Val Gly Gly Ser Ile Thr Tyr Asp
        195                 200                 205

Tyr Glu Gly Phe Gly Ile Gly Gly Ala Ile Ser Ser Ser Lys Arg Thr
    210                 215                 220

Asp Ala Gln Asn Thr Ala Ala Tyr Ile Gly Asn Gly Asp Arg Ala Glu
225                 230                 235                 240

Thr Tyr Thr Gly Gly Leu Lys Tyr Asp Ala Asn Asn Ile Tyr Leu Ala
                245                 250                 255

Ala Gln Tyr Thr Gln Thr Tyr Asn Ala Thr Arg Val Gly Ser Leu Gly
            260                 265                 270

Trp Ala Asn Lys Ala Gln Asn Phe Glu Ala Val Ala Gln Tyr Gln Phe
        275                 280                 285

Asp Phe Gly Leu Arg Pro Ser Leu Ala Tyr Leu Gln Ser Lys Gly Lys
    290                 295                 300

Asn Leu Gly Arg Gly Tyr Asp Asp Glu Asp Ile Leu Lys Tyr Val Asp
305                 310                 315                 320

Val Gly Ala Thr Tyr Tyr Phe Asn Lys Asn Met Ser Thr Tyr Val Asp
                325                 330                 335

Tyr Lys Ile Asn Leu Leu Asp Asp Asn Gln Phe Thr Arg Asp Ala Gly
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Ile Asn Thr Asp Asn Ile Val Ala Leu Gly Leu Val Tyr Gln Phe
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             20                  25                  30

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         35                  40                  45

Lys Ala Val Ala Ala Ser Lys Glu Arg Asn Gly Leu Ser Leu Ala Ala
     50                  55                  60

Leu Lys Lys Ala Leu Ala Ala Gly Gly Tyr Asp Val Glu Lys Asn Asn
 65                  70                  75                  80

Ser Arg Ile Lys Leu Gly Leu Lys Ser Leu Val Ser Lys Gly Thr Leu
                 85                  90                  95

Val Gln Thr Lys Gly Thr Gly Ala Ser Gly Ser Phe Lys Leu Asn Lys
            100                 105                 110

Lys Ala Ala Ser Gly Glu Ala Lys Pro Lys Ala Lys Lys Ala Gly Ala
        115                 120                 125

Ala Lys Ala Lys Lys Pro Ala Gly Ala Thr Pro Lys Lys Ala Lys Lys
    130                 135                 140

Ala Ala Gly Ala Lys Lys Ala Val Lys Lys Thr Pro Lys Lys Ala Lys
145                 150                 155                 160

Lys Pro Ala Ala Ala Gly Val Lys Lys Val Ala Lys Ser Pro Lys Lys
                165                 170                 175

Ala Lys Ala Ala Ala Lys Pro Lys Lys Ala Thr Lys Ser Pro Ala Lys
            180                 185                 190

Pro Lys Ala Val Lys Pro Lys Ala Ala Lys Pro Lys Ala Ala Lys Pro
        195                 200                 205

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Lys Lys
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225

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Val Asp Gly

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Gly Thr Gly Ala Ser Gly Ser Phe Lys Leu Asn Lys Lys Ala Ala Ser
35 40 45

Gly Glu Ala Lys Pro Lys Ala Lys Lys Ala Gly Ala Ala Lys Ala Lys
50 55 60

Lys Pro Ala Gly Ala Thr Pro Lys Lys Ala Lys Lys Ala Ala Gly Ala
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Lys Lys Ala Val Lys Lys Thr Pro Lys Lys Ala Lys Lys Pro Ala Ala
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Ala Lys Pro Lys
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20 25 30

Gly Thr Gly Ala Ser Gly Ser Phe Lys Leu Asn Lys Lys Ala Ala Ser
35 40 45

Gly Glu Ala Lys Pro Lys Ala Lys Lys Ala Gly Ala Ala Lys Ala Lys
50 55 60

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Ala Gly Val Lys Lys Val Ala
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35 40 45

Gly Glu Ala Lys Pro Lys Ala Lys Lys Ala Gly Ala Ala Lys Ala Lys
50 55 60

Lys Pro Ala Gly Ala Thr Pro Lys Lys Ala Lys Lys Ala Ala Gly Ala
65 70 75 80

Lys Lys Ala Val Lys Lys Thr Pro Lys Lys Ala Lys Lys Pro Ala Ala
85 90 95

Ala Gly Val Lys Lys Val Ala Lys Ser Pro Lys Lys Ala Lys Ala Ala
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Ser Phe Leu Ala Ser Tyr Arg
1 5

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Ser Phe Leu Ser Ala Ser Tyr Arg

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ASKERSGVSLAALKKALAAAGYDVEKNNSRIKLGLKSLVSKGTLVQTK
GTGASGSFKLNKKAASGEAKPKVKKAGGTKPKKPVGAAKKPKKAAG
GATPKKSAKKTPKKAKKPAAATVTKKVAKSPKKAKVAKPKKAAKSA
AKAVKPKAAKPKVVKPKKAAPKKK

Human Histone $H1^{S}$-2 (SEQ ID NO: 2)
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ASKERSGVSLAALKKALAAAGYDVEKNNSRIKLGLKSLVSKGTLVQTK
GTGASGSFKLNKKAASGEGKPKAKKAGAAKPRKPAGAAKKPKKVAG
AATPKKSIKKTPKKVKKPATAAGTKKVAKSAKKVKTPQPKKAAKSPA
KAKAPKPKAAKPKSGKPKVTKAKKAAPKKK

Human Histone $H1^{S}$-3 (SEQ ID NO: 3)
SETAPAETATPAPVEKSPAKKKATKKAAGAGAAKRKATGPPVSELITK
AVAASKERNGLSLAALKKALAAGGYDVEKNNSRIKLGLKSLVSKGTL
VQTKGTGASGSFKLNKKAASGEAKPKAKKAGAAKAKKPAGATPKKA
KKAAGAKKAVKKTPKKAKKPAAAGVKKVAKSPKKAKAAAKPKKAT
KSPAKPKAVKPKAAKPKAAKPKAAKPKAKKAAAKKK

Human Histone $H1^{S}$-4 (SEQ ID NO: 4)
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ASKERSGVSLAALKKALAAAGYDVEKNNSRIKLGLKSLVSKGTLVQTK
GTGASGSFKLNKKAASGEAKPKAKKAGAAKAKKPAGAAKKPKKATG
AATPKKSAKKTPKKAKKPAAAAGAKKAKSPKKAKAAKPKKAPKSPA
KAKAVKPKAAKPKTAKPKAAKPKKAAAKKK

Human Histone $H1^{\circ}$ (SEQ ID NO: 5)
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DEPKKSVAFKKTKKEIKKVATPKKASKPKKAASKAPTKKPKATPVKKA
KKKLAATPKKAKKPKTVKAKPVKASKPKKAKPVKPKAKSSAKRAGK
KK

Human Histone H1t (SEQ ID NO: 6)
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ALSVSQERVGMSLVALKKALAAAGYDVEKNNSRIKLSLKSLVNKGILV
QTRGTGASGSFKLSKKVIPKSTRSKAKKSVSAKTKKLVLSRDSKSPKTA
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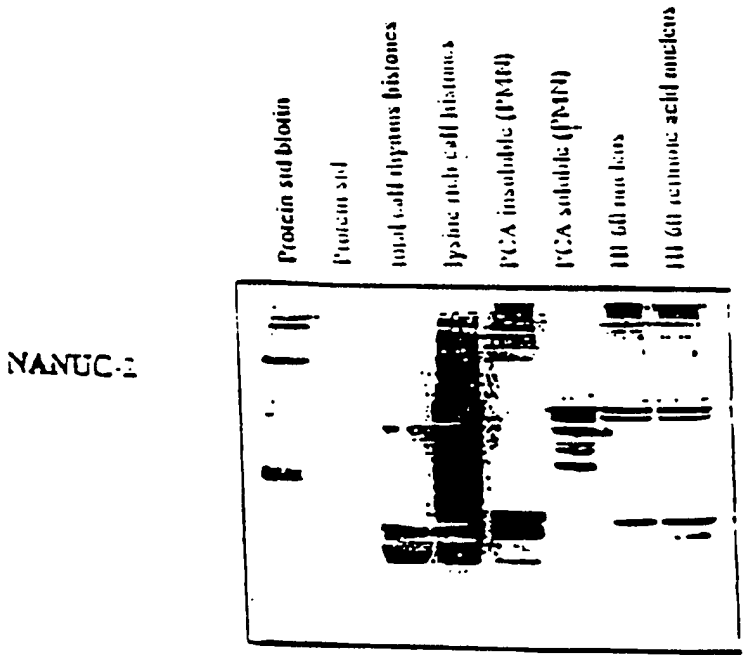
Figure 1



NANUC-2

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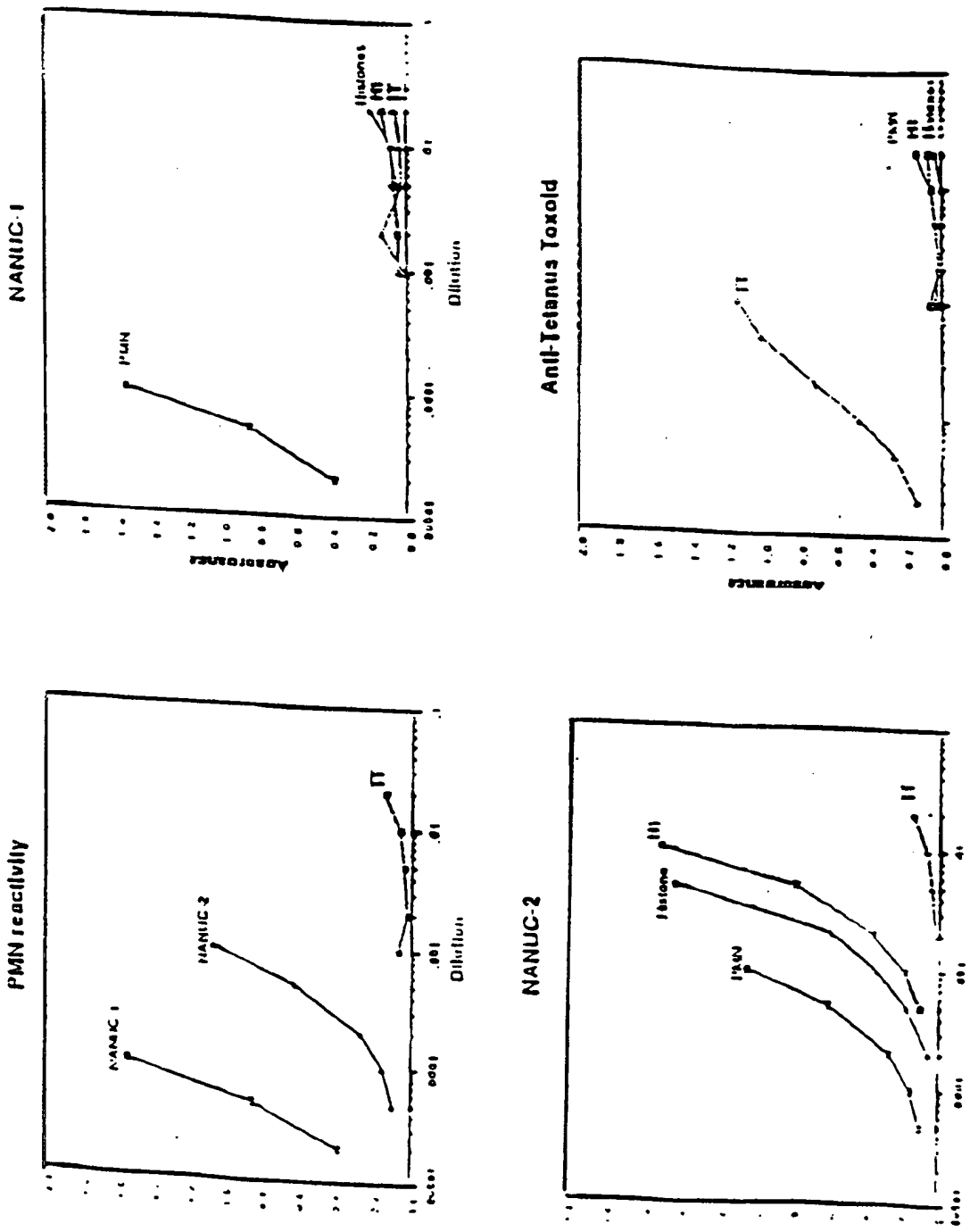
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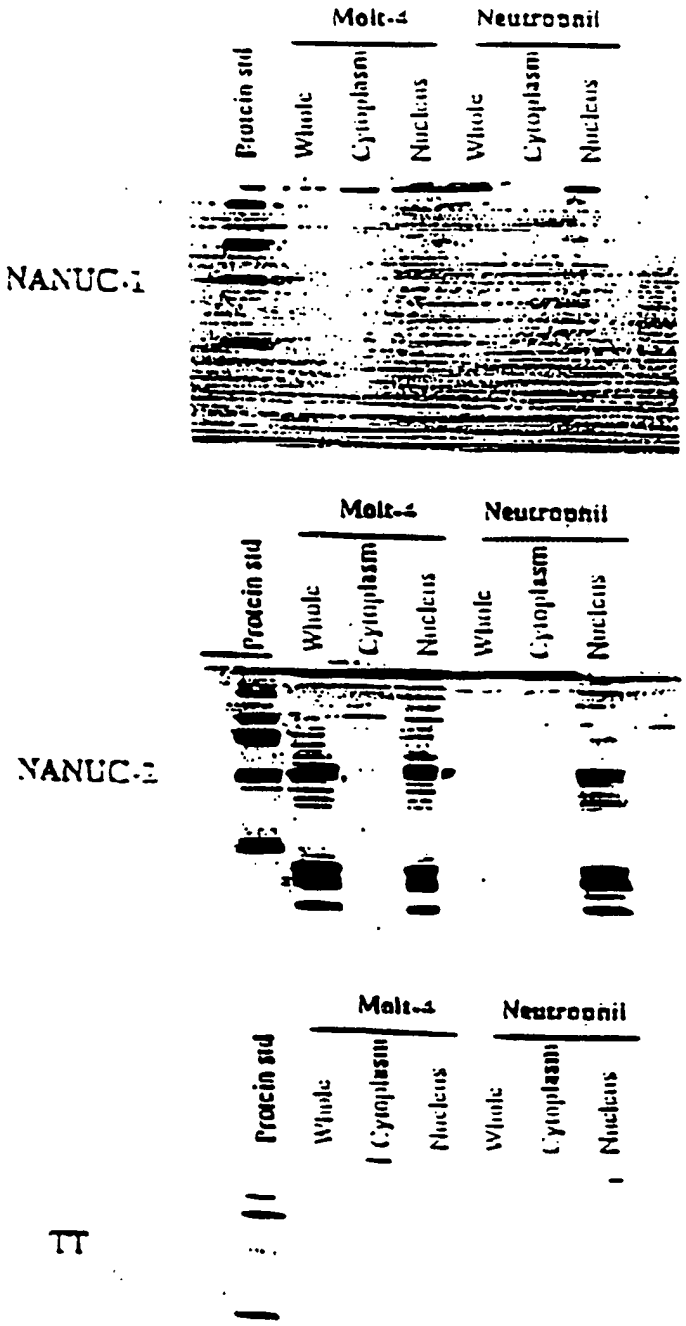
PMN
Histones
H1
H2
H3
H4
Dilution
Anti-Tetanus Toxoid
PMN reactivity
NANUC-1
NANUC-2
NANUC-2

Figure 3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Neutrophil
Protein std
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Protein std
Whole
Cytoplasm
Nucleus
Whole
Cytoplasm
Nucleus
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Protein std
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Cytoplasm
Nucleus
Whole
Cytoplasm
Nucleus
TT

Figure 4

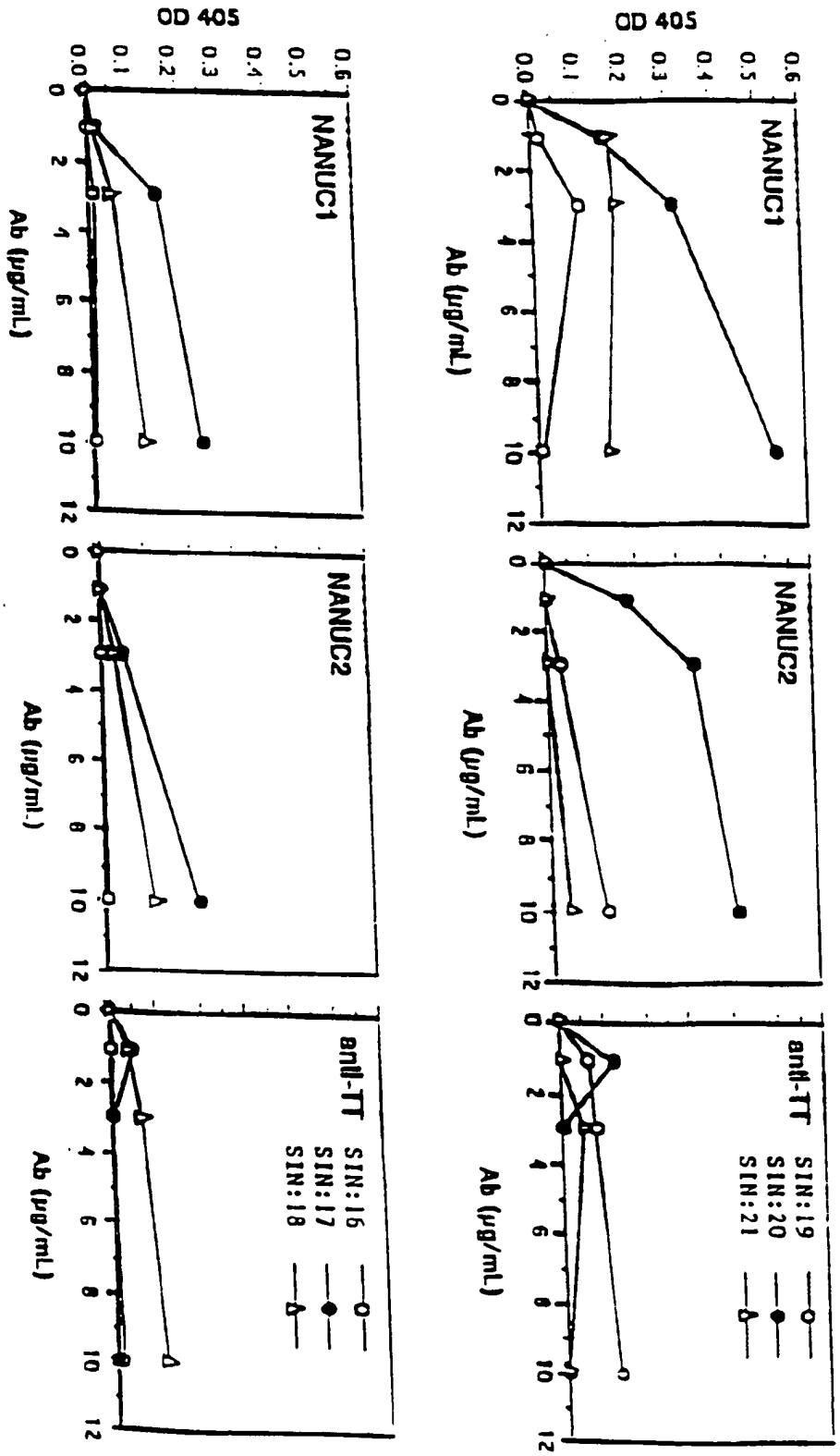
OD 405
NANUC1
NANUC2
anti-TT
Ab (µg/mL)
SIN:16
SIN:17
SIN:18
SIN:19
SIN:20
SIN:21

Figure 5

STRAIN	SOURCE	STOCK	MEDIUM	GROWTH
Mycobacterium tuberculosis (Erdman -Pathogenic)	ATCC #35801	-70°C 8% Glycerol	Middlebrook 7H9 Medium with OADC Enrichment plus: 1g L-glutamine/L. and 1% Glycerol see ATCC#173	~3 weeks (18-21days), at 37°C with 5% CO_2 from $5x10^5$ cfu/ml to 1-$2x10^8$ cfu/ml in 300ml volume/2L flask
Mycobacterium bovis (Pathogenic)	ATCC #19210	-70°C 8% Glycerol	Middlebrook 7H9 Medium with OADC Enrichment plus: 1g L-glutamine/L. and 1% Glycerol see ATCC#173	~3 weeks (18-21days), at 37°C with 5% CO_2 from $5x10^5$ cfu/ml to 1-$2x10^8$ cfu/ml in 300ml volume/2L flask
Mycobacterium bovis BCG (Attenuated)	ATCC #35734	-70°C 8% Glycerol	Middlebrook 7H9 Medium with OADC Enrichment plus: 1g L-glutamine/L. and 1% Glycerol see ATCC#173	~3 weeks (18-21days), at 37°C with 5% CO_2 from $5x10^5$ cfu/ml to 1-$2x10^8$ cfu/ml in 300ml volume/2L flask
Mycobacterium smegmans 1-2c (Non-pathogen)	Douglas Young, St.Mary's Hospital Medical School, London. UK.	-70°C 8% Glycerol	Middlebrook 7H9 Medium with OADC Enrichment plus: 1g L-glutamine/L. and 1% Glycerol see ATCC#173	3 days, at 37°C with 5% CO_2 on shaker at 180rpm, from $5x10^5$ cfu/ml to 2.5-$x10^8$ cfu/ml in 300ml volume/4L flask
Mycobacterium avium (Opportunistic Pathogen)	ATCC #25291	-70°C 8% Glycerol	Middlebrook 7H9 Medium with OADC Enrichment plus: 1g L-glutamine/L. and 1% Glycerol see ATCC#173	~3 weeks (18-21days), at 37°C with 5% CO_2 from $5x10^5$ cfu/ml to 1-$2x10^8$ cfu/ml in 300ml volume/2L flask
Mycobacterium avium Paratuberculosis	ATCC #19698	-70°C 8% Glycerol	Middlebrook 7H9 Medium with OADC Enrichment plus: 1g L-glutamine/L. 1% Glycerol, and 70µM FeCl, see ATCC#173	~3 weeks (18-21days), at 37°C with 5% CO_2 from $5x10^5$ cfu/ml to 1-$2x10^8$ cfu/ml in 300ml volume/2L flask
Mycobacterium avium paratuberculosis "Linda"strain (Crohn's patient isolate)	ATCC #43015	-70°C 8% Glycerol	Middlebrook 7H9 Medium with OADC Enrichment plus: 1g L-glutamine/L. 1% Glycerol, and 70uM FeCl, see ATCC#173	~3 weeks (18-21days), at 37°C with 5% CO_2 from $5x10^5$ cfu/ml to 1-$2x10^8$ cfu/ml in 300ml volume/2L flask

Figure 6

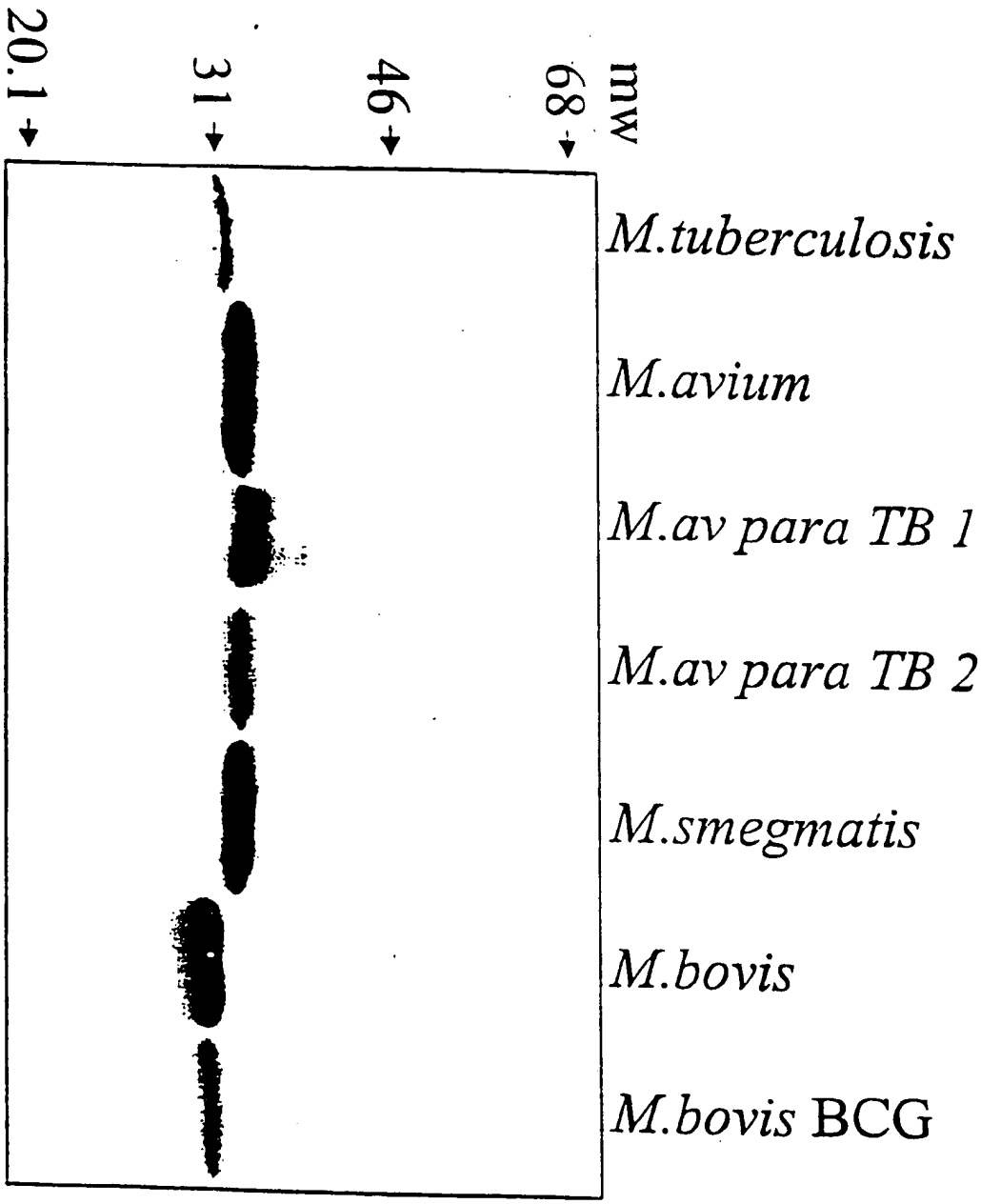
20.1
31
46
68
mw
M.tuberculosis
M.avium
M.av para TB 1
M.av para TB 2
M.smegmatis
M.bovis
M.bovis BCG

Figure 7

```
                1                15 16            30 31            45 46            60 61            75 76            90
1 H-term MHKAELIDVLTTKHX SDR----RQXTAXVE D----------------------------------------------------------------------
2 214    MHKAELIDVLTQKLG SDR----RQATAAVE NVVDTIVRAVHKGDS VTITGFGVFEQRR-- -------RAARVARN PRTGETVKVKPTSVP
3 111.5  MSETAPAETATPAPV EKS-PAKKKATKKAA GAGAAKRKATGPPVS ELITKAVAASKERNG LSLAALKKALAAGGY DVEKNNSRIKLGLKS

                91               105 106          120 121          135 136          150 151          165 166          180
1 H-term ------------------------------------------------------------------------------------------------------------
2 214    AFRPGAQFKAVVSGA QRLPAEGPAVKRGVG ASAAKKVAKKAPAKK ATKAAKKAATKAPAR KAATKAPAKKAATKA PAKKAVKATKSPAKK
3 111.5  IVSKGTLVQTKGTGA SGSFKLNKKAASGEA KPKAKKAGAAKAKKP AGATPKKAKKAAGAK KAVKKTPKKAKKPAA AGVKKVAKSPKKAKA

                181              195 196          210 211          225 226
1 H-term ------------------------------------------ --                  27
2 214    VTKAVKKTAVKASVR KAATKAPAKKAAAKH PATKAPAKKATARRG RK              214
3 111.5  AAKPKKATKSPAKPK AVKPKAAKPKAAKPK AAKPKAAKAKKAAAK KK              226
```

Figure 8

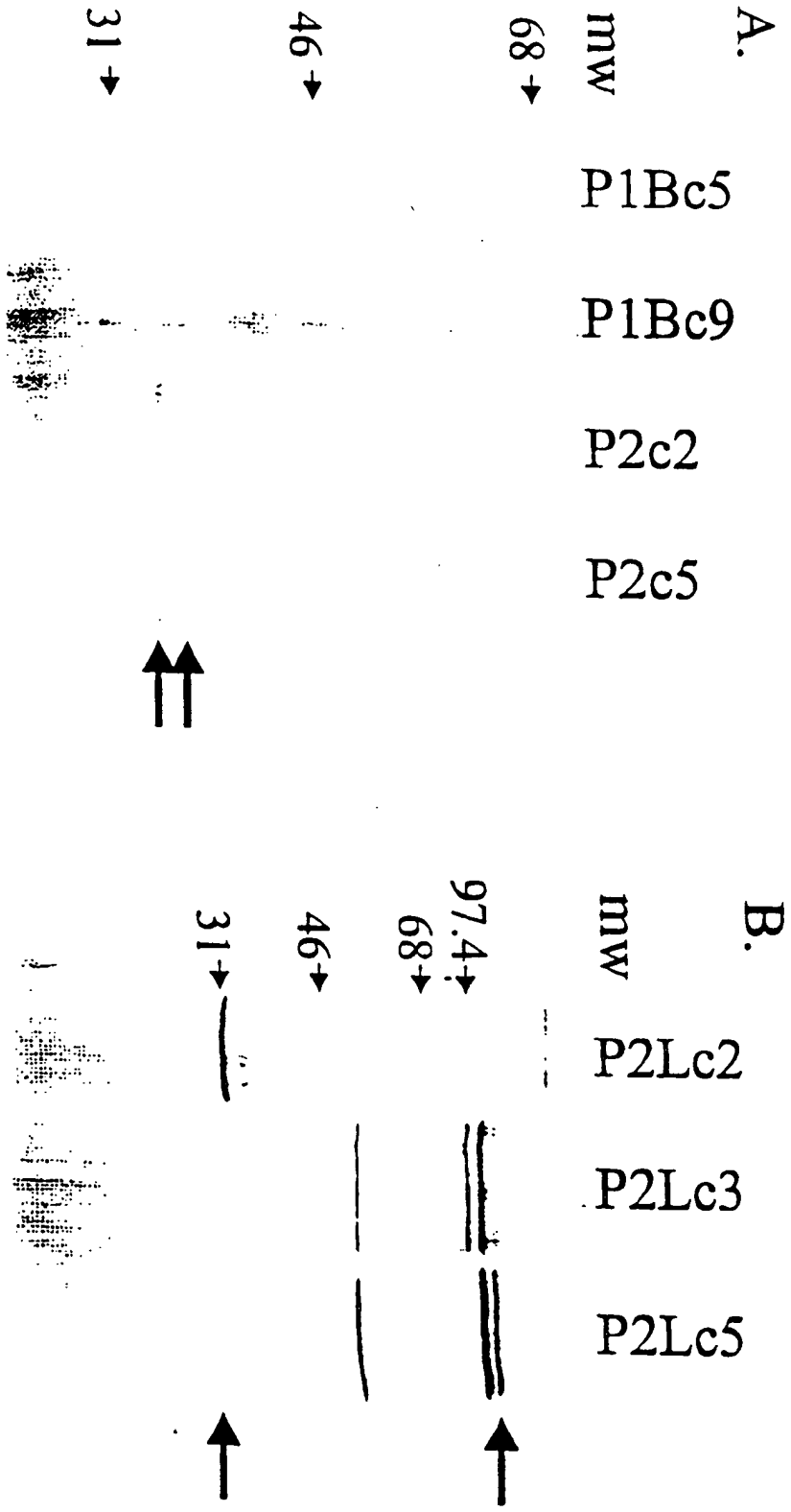
A.
mw
68
46
31
P1Bc5
P1Bc9
P2c2
P2c5
B.
mw
97.4
68
46
31
P2Lc2
P2Lc3
P2Lc5

Figure 9

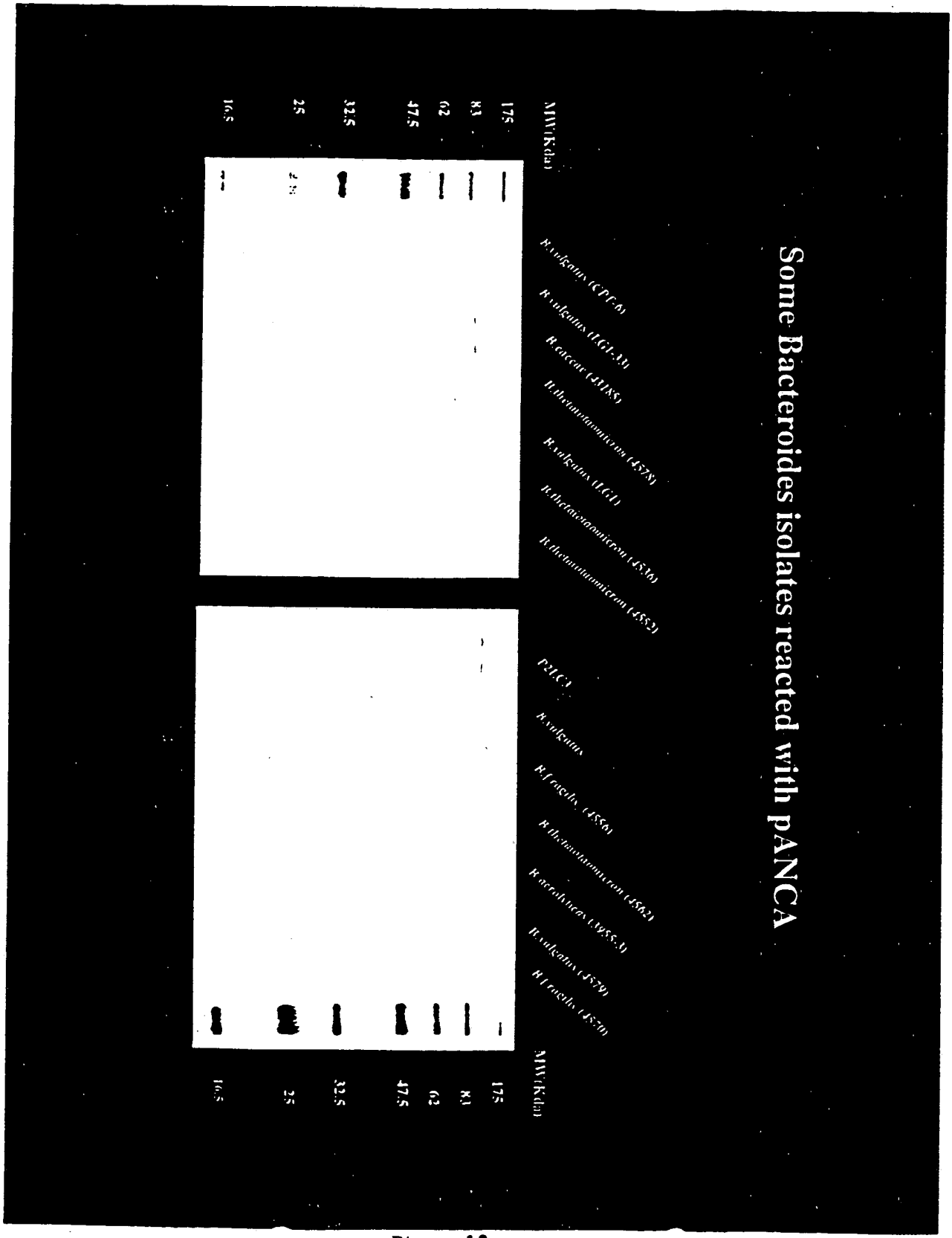
Some Bacteroides isolates reacted with pANCA
MW(Kda)
175
83
62
47.5
32.5
25
16.5
B.vulgatus (CPTC-A)
B.vulgatus (LGT-33)
B.caccae (45185)
B.thetaiotaomicron (4578)
B.vulgatus (LG1)
B.thetaiotaomicron (4536)
B.thetaiotaomicron (4552)
P2LC-1
B.vulgatus
B.fragilis (4556)
B.thetaiotaomicron (4562)
B.ovatus (4555-3)
B.vulgatus (4579)
B.fragilis (4570)

Figure 10.

Human histone H1a (H1.5):

```
  1 msetapaeta tpapvekspa kkkatkkaag agaakrkatg ppvselitka vaaskerngl
 61 slaalkkala aggydveknn sriklglksl vskgtlvqtk gtgasgsfkl nkkaasgeak
121 pkakkagaak akkpagatpk kakkaagakk avkktpkkak kpaaagvkkv akspkkakaa
181 akpkkatksp akpkavkpka akpkaakpka akpkaakakk aaakkk
```

M.tubeculosis 214 amino acid histone H1 homologue:

```
  1 mnkaeiidvl tqklgsdrrq ataavenvvd tivravnkgd svtitgfgvf eqrrraarva
 61 rnprtgetvk vkptsvpafr pgaqfkavvs gaqrlpaegp avkrgvgasa akkvakkapa
121 kkatkaakka atkaparkaa tkapakkaat kapakkavka tkspakkvtk avkktavkas
181 vrkaatkapa kkaaakrpat kapakkatar rgrk
```

E. coli 323 amino acids outer membrane protein F precursor:

```
  1 mkskvialli pallaagaah aaevynkdgn kldlygkvdg lhyfsdnsak dgdqsyarlg
 61 fkgetqindq ltgyggqweyn iqanntessk nqswtrlafa glkfadygsf dygrnygvmy
121 diegwtdmlp efggdsytna dnfmtgrang vatyrntdff glvnglnfav qyqgnnegas
181 ngqegtnngr dvrhengdgw glsttydlgm gfsagaayts sdrtndqvnh taaggdkada
241 wtaglkydan niylatmyse trnmtpfgds dyavanktqn fevtaqyqfd fglrpavsfl
301 mskgrdlhaa ggadnpagvd dkd
```

E. coli 377 amino acids outer membrane protein F precursor:

```
  1 mkskvialli pallaagaah aaevynkdgn kldlygkvdg lhyfsdnsak dgdqsyarlg
 61 fkgetqindq ltgyggqweyn iqanntessk nqswtrlafa glkfadygsf dygrnygvmy
121 diegwtdmlp efggdsytna dnfmtgrang vatyrntdff glvnglnfav qyqgnnegas
181 ngqegtnngr dvrhengdgw glsttydlgm gfsagaayts sdrtndqvnh taaggdkada
241 wtaglkydan niylatmyse trnmtpfgds dyavanktqn fevtaqyqfd fglrpavsfl
301 mskgrdlhaa ggadnpagvd dkdlvkyadi gatyyfnknm styvdykinl ldeddsfyaa
361 ngistddiva lglvyqf
```

E. coli 367 amino acids outer membrane protein c precursor:

```
1 mkvkvlsllv pallvagaan aaevynkdgn kldlygkvdg lhyfsdnkdv dgdqtymrlg
 61 fkgetqvtdq ltgyggqweyq iqgnsaenen nswtrvafag lkfqdvgsfd ygrnygvvyd
121 vtswtdvlpe fggdtygsdn fmqqrgngfa tyrntdffgl vdglnfavqy qgkngnpsge
181 gftsqvtnng rdalrqngdg vggsltydye gfgiggaiss skrtdaqnta ayignggrae
241 tytggikyda nniylaaqyt qtynatrvgs lgwankaqnf eavaqyqfdf girpslaylq
301 skgknlgrgy ddedilkyvd vgatyyfnkn mstyvdykin llddnqftrd agintdniva
361 lglvyqf
```

Figure 11

```
                1                15 16            30 31            45 46            60 61            75 76            90
N               ---------------------AEVYNKDGN KLDLYGKVDG----- ---------------- ---------------- ---------------- ----------
323 MKSKVLALLIPALLA AGAAHAAEVYNKDGN KLDLYGKVDGLHYFS DNSAKDGDQSYARLG FKGETQINDQLTGYG QWEYNIQANNTESSK
h1.5 ----------MSETA PAETATPAPVEKSPA KKKATKKAAGAGAAK RKATGPPVSELITKA VAASKERNGLSLAAL KKALAAGGYDVEKNN

               91               105 106          120 121          135 136          150 151          165 166          180
N               ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
323 NQSNTRLAFAGLKFA DYGSFDYGRNYGVHY DIEGWTDMLPEFGGD SYTNADNFMTGRANG VATYRNTDFFGLVNG LNFAVQYQGNNEGAS
h1.5 SRIKLGLKSLVSKGT LVQTKGTGASGSFKL NKRAASGEAKPKAKK AGAAKAKKPAGATPK KAKKAAGAKKAVKKT PKKAKKPAAAGVKKV

               181              195 196          210 211          225 226          240 241          255 256          270
N               ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
323 NGQEGTNNGRDVRHE NGDGWGLSTTYDLGM GFSAGAAYTSSDRTN DQVNHTAAGGDKADA WTAGLKYDANNIYLA TMYSETRNMTPFGDS
h1.5 AKSPKKAKAAAKPKK ATKSPAKPKAVKPKA AKPKAAKPKAAKPKA AKAKKAAAKKK---- ---------- ---------- ----------

               271              285 286          300 301          315 316
N               ---------- ---------- ---------- ---------- --------
323 DYAVANKTQNFEVTA QYQFDFGLRPAVSFL NSKGRDLHAAGGADN PAGVDDKD
h1.5 ---------- ---------- ---------- ---------- --------
```

Figure 12

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/05492

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61K 39/00, 39/02, 39/385; C12Q 1/18; G01N 33/564, 33/569
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/184.1, 190.1, 193.1, 234.1, 278.1; 435/7.21, 7.24, 7.32, 32; 436/506

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Please See Extra Sheet.

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X ---- Y	WO97/38713 A1 (THE REGENTS OF THE UNIVERSITY OF CALIFORNIA) 23 October 1997. See claims 1-24.	1,3,5 ------- 2,4,6
X ---- Y	EGGENA ET AL, Histone H1: the ulcerative colitis specific pANCA target antigen, FASEB Journal, 30 April 1996, Volume 10, Number 6, page A1079, abstract number 463. See entire abstract.	1-2 ----- 4,6
X ---,P Y	COHAVY ET AL, Identification of candidate bacterial pathogens in ulcerative colitis using a disease-specific marker antibody, FASEB Journal, 17 March 1998, Volume 12, Number 4, page A593, abstract 3446. See entire abstract.	13-14 -------- 19-23

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 23 JUNE 1999	Date of mailing of the international search report 07 JUL 1999
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer DAVID A SAUNDERS Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/05492

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☒ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/05492

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	SARTOR, Pathogenesis and immune mechanisms of chronic inflammatory bowel diseases, American Journal of Gastroenterology, December 1997, Volume 92, Number 12, pages 5S-11S. See page 9S, paragraph spanning columns 1-2.	19-23

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/05492

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

424/184.1, 190.1, 193.1, 234.1, 278.1; 435/7.21, 7.24, 7.32, 32; 436/506

B. FIELDS SEARCHED
Documentation other than minimum documentation that are included in the fields searched:

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

BIOSIS

(porin or ompf or ompc or channel) and (uc or ulcerat?(W)colitis)

bacteroides and (ulcerat?(W)colitis or uc or panca or p(W)anca)

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claim(s)1-2, drawn to diagnostic method employing histone H-1 antigen.
Group II, claim(s) 3-6, drawn to treatment method employing histone H-1 antigen.
Group III, claim(s) 7-8, drawn to diagnostic method employing porin antigen.
Group IV, claim(s) 9-12, drawn to treatment method employing porin antigen.
Group V, claim(s) 13-14, drawn to diagnostic method employing Bacteroides antigen.
Group VI, claim(s) 15-18, drawn to treatment method employing Bacteroides antigen.
Group VII, claim(s) 19-23, drawn to method of screening for anti-bacterial agents.

The inventions listed as Groups I-VII do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

The antigens used in the diagnostic methods of Groups I, III and V differ from each other with respect to their sources and structures. A separate search is required for each in both the patent and non-patent scientific literature, and a teaching or suggestion of one method in the literature need not suggest any of the others. Each method would thus constitute a separate contribution over the prior art and therefor does not have the same or corresponding special technical feature as that of the other Groups.

The antigens used in the treatment methods of Groups II, IV and VI differ from each other with respect to their sources and structures. A separate search is required for each in both the patent and non-patent scientific literature, and a teaching or suggestion of one method in the literature need not suggest any of the others. Each method would thus constitute a separate contribution over the prior art and therefor does not have the same or corresponding special technical feature as that of the other Groups.

While the antigen use in the diagnostic and treatment methods of Groups I and II is the same, these methods are separate contributions over the art in that they have no common steps and can be practiced independently of one another. For example, the treatment method of Group II could be practiced after diagnosis of UC via another method or combination of methods, such as the detection of pANCA reactivity against neutrophils. Thus these two Groups do not have the same or corresponding special technical feature. Like considerations apply to the diagnostic and treatment methods of Groups III and IV and of Groups V and VI.

The agent identifying or screening method of Group VII, identifies no agent that is used in the diagnostic or treatment

methods of Groups I-VI, and the agent would have no structural features or mechanism of action in common with the agents used in the treatment methods of Groups II, IV or VI. The agent identified could be used to treat UC after diagnosis via methods other than those of Groups I, III or V, as noted in the above paragraph. This Group thus constitutes a separate contribution over the prior art and does not have the same or corresponding special technical feature of any other Group.